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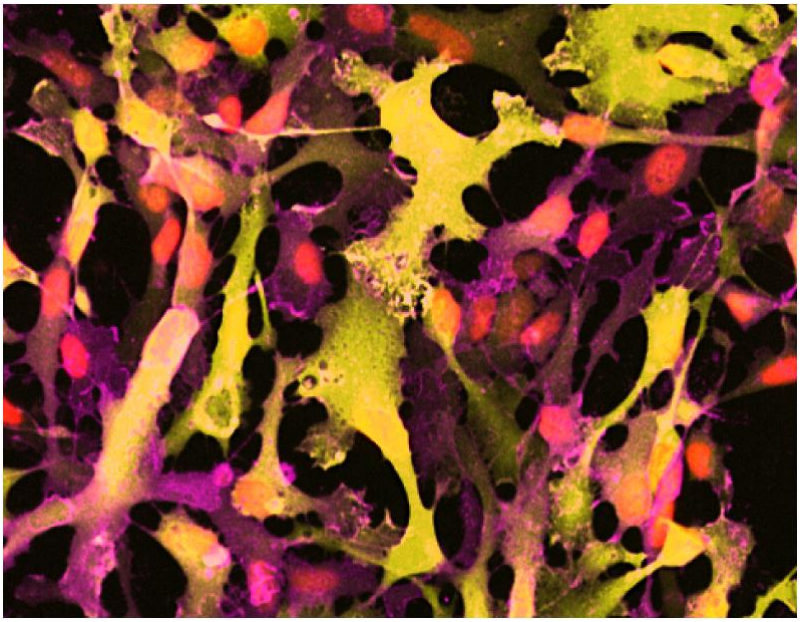
HUMAN IPSC DERIVED NEURAL CELLS AS MODELS OF BRAIN DEVELOPMENT AND AS TOOLS IN PHARMACEUTICAL DRUG DISCOVERY



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Cover illustration: Human induced pluripotent stem cell derived astroglia expressing key proteins of astrocyte development showing cytoplasmic protein FABP7 (yellow), astrocyte associated glutamate transporter SLC1A3 (purple) and transcription factor SOX9 (red).

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Human iPSC derived neural cells as models of brain development and as tools in pharmaceutical drug discovery

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To Hanna

My friend
My partner
My love and inspiration

ABSTRACT

Human brain evolution has resulted in a cognitive superiority compared to all other animals. Unique cortical structures and expanding progenitor populations have been associated with the possibility for developing a highly folded neocortex and expanded surface area, which is linked to cognitive function. Alongside the development of the neuronal population there has been a remarkable evolution of a second population of brain cells called astrocytes. Astrocytes, which historically have been viewed as the glue of the brain, are now considered as a major regulator of brain homeostasis and neuron communication. Hypothesized to meet the increased complexity of neuronal sub-populations astrocytes have become highly diversified. Specific astrocytes can only be observed in higher primates and generally comprises a more advance form and structure enabling a single astrocyte to support a higher number of neurons. Additionally, it has been shown that human astrocytes can improve cognitive function in mice, an observation signifying the importance of astrocytes in human brain evolution. However, increased complexity is accompanied by biological errors resulting in human specific diseases. Disease mechanisms linked to human biological traits poses challenges when trying to uncover and develop treatments against its pathological conditions using animal models. With decreasing drug developmental programs in the pharmaceutical industry targeting neurological and psychiatric diseases there is a need to improve and accelerate drug discovery in this area.

Studying cellular functions of the human brain is challenging partly due to limited accessibility of brain tissue. Historically, the main source of cells was derived from healthy tissue following surgical procedures as well as post-mortem and fetal tissue. However, since the discovery of induced pluripotent stem cells, having the potential to generate any cell type in the body, accessibility to neural like cells has changed dramatically. Common strategies for acquiring neurons and astrocytes from pluripotent stem cells are to try and mimic the naturally occurring embryonic development. However, this requires the establishment of defined and detailed protocols instructing the cells how to develop and becoming the cell type of interest. Neurons follow a step-wise development program which have been uncovered and in great parts mimicked in the lab. However, whether this step-wise developmental progression holds true for astrocytes is yet to be defined.

The aim of this thesis was to develop a protocol to derive astrocytes from human induced pluripotent stem cells (hiPSC) and benchmark them against current models available for the pharmaceutical industry. Moreover, the project aimed to establish hiPSC derived neuronal and astrocyte models in a pharmaceutical setting to investigate their potential contribution in drug development.

The characterization of four astrocytic models in comparison to a neural stem cell and non-neural model showed expected astrocyte specific characteristics. However, large differences in gene expression and astrocyte associated functions indicated a large heterogeneity among models which was also demonstrated in drug response stimulations. This clearly implies that discovery of new chemical compounds for further drug development will be context dependent,

having identification bias towards the model of choice. Moreover, thorough characterization and diverse applications demonstrated a very robust and reproducible protocol for the generation of hiPSC derived astrocytes, a feature naturally critical if utilized in pharmaceutical assays. Finally, in addition to improved functionality compared to conventional models, hiPSC derived astrocytes show developmental traits linked to embryonic development increasing translability and model relevance.

Furthermore, in a proof of principle study hiPSC derived neurons were shown to be able to predict unwanted side effect of a drug used to prevent excessive blood loss from major trauma or surgery. The drug is believed to affect specific neurons resulting in involuntary seizures. Besides demonstrating receptor activity of the drug, human iPSC derived neurons were shown to be applicable in the development of new drugs lacking this side effect. Finally, this was performed using a label-free and simple method which is highly applicable for drug screening.

In conclusion this thesis presents a protocol for the derivation of an astrocytic model having translatability to the embryonic development and possesses several cellular functions observed by astrocytes *in vivo*. The application of hiPSC derived neurons and astrocytes in a pharmaceutical setting demonstrate that they can make a significant contribution in drug discovery.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following studies reffered to in text by their roman numnbers.

I. **Lundin, A.**, Delsing, L., Clausen, M., Ricchiuto, P., Sanchez, J., Sabirsh, A., Ding, M., Synnergren, J., Zetterberg, H., Brolén, G. Hicks, R., Herland, A., Falk, A.
Human iPS-Derived Astroglia from a Stable Neural Precursor State Show Improved Functionality Compared with Conventional Astrocytic Models.
Stem Cell Reports. doi:10.1016/j.stemcr.2018.01.021

II. Kristensson L.*, **Lundin A.***, Gustafsson, D., Fryklund, J., Fex, T., Delsing, L., Ryberg, E.
Plasminogen binding inhibitors demonstrate unwanted activities on GABAA and glycine receptors in human iPSC derived neurons.
Neuroscience Letters. doi.org/10.1016/j.neulet.2018.05.018

III. **Lundin, A.**, Ricchiuto, P., Clausen, M., Hicks, R., Falk, A., Herland, A.
Directed hiPS-derived astroglia model show temporal transcriptional transition of long- and small-RNAs associated with glia competence acquisition.
(Manuscript)

* Equal contribution

1. CONTENTS

LIST OF ABBREVIATIONS

ACh	Acetylcholine
AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
AP	Anterior-posterior
Aβ	Amyloid-β
BG	Bergmann glia
BMP	Bone morphogenetic protein
CNS	Central nervous system
CP	Cortical plate
DMR	Dynamic mass redistribution
DV	Dorsal-ventral
EB	Embryonic body
ER	Endoplasmic reticulum
FACS	Fluorescent activated cell sorting
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
GABA	gamma-Aminobutyric acid
GCPR	G protein-coupled receptors
GRP	Glia restricted progenitor cells
GW	Gestation week
HTS	High-through put screening
IP	Intermediate progenitor
IZ	Intermediate zone
JAK	Janus kinases
KO	Knock-out
LGE	Lateral ganglionic eminence
MEA	Multielectrode array
MGE	Medial ganglionic eminence
MPEP	2-methyl-6-phenylethynyl-pyridine (GRM5 antagonist)
MS	Multiple sclerosis
MZ	Marginal zone
NMDA	N-methyl-D-aspartate receptor
NPC	Neural progenitor cells
NSC	Neural stem cells
OPC	Oligodendrocyte progenitor cells
PAP	Peripheral astrocyte processes
PBI	Plasminogen binding inhibitor
PD	Parkinson's disease
PLO	poly-L-ornithine
PNS	Peripheral nervous system
RA	Retinoic acid
RG	Radial glia
SHH	Sonic hedgehog
SP	Subplate
STAT	Signal transducer and activator of transcription proteins
SVZ	Sub ventricular zone
VA	Velate astrocyte
VZ	Ventricular zone
WNT	Wingless
hESC	Human embryonic stem cells
hPSC	Human pluripotent stem cells
hiPSC	Human induced pluripotent stem cells
iSVZ	Inner SVZ
lncRNA	Long non-coding RNA
miRNA	Micro RNA
ncRNA	Non-coding RNA
oRG	Outer RG
oSVZ	Outer SVZ
tRG	Truncated RG
vRG	Ventricular RG

Gene names follow HUGO gene nomenclature committee (HGNC) guidelines and common synonyms are shown in brackets [].

1 Introduction

Studying brain development and neural cellular function of human origin has traditionally been performed using isolated cells from human primary tissue of post-mortem samples, healthy tissue from surgical procedures and fetal tissue. However, primary isolation is always associated with limited accessibility which has been addressed by isolation, expansion and banking of stem cells or the generation of immortalized cell lines. Human embryos have historically been the only source for human pluripotent stem cells (hPSC) which due to ethical concerns and limited access have been applied sparsely. The emergence of the induced pluripotent stem cell (iPSC) technology (6) enabled the generation of human pluripotent stem cells from various sources such as skin, and consequently, the field has developed tremendously. *In vitro* development of specific somatic cells demonstrates impressive similarities to human embryonic development. When differentiated, hiPSC follow embryonic germline specification and regional patterning all along the neural tube and subsequent niche specific differentiation.

Compared to astrocytic models hiPSC derived neuronal models have developed much faster with established protocols generating subspecific neurons and identities of specific cortical layers (7). The time for hiPSC to acquire neuronal cell fate is shorter for neurons than for astrocytes, a feature which correlates with human embryonic development. This highlights that culturing time is a possible factor for the observed differences in technical development of neuronal and astrocytic hiPSC models. During embryonic development astrocytes preced the development for oligodendrocytes, however, there are robust hiPSC-protocols established for the latter (8, 9). Neurons and oligodendrocytes follow a step-wise development where progenitors are restricted towards a specific lineage. The cells subsequently migrate from their regional centers and, upon arrival at the target region, exit the cell cycle and undergo terminal differentiation. Whether this step-wise developmental progression holds true for the heterogeneity of astrocytes has yet to be defined (10). Incomplete understanding of specific astrocytic developmental processes is in part due to the lack of reliable markers to characterize progenitors and astrocytes during development in contrast to what has been established for oligodendrocytes (11). Additionally, as a large fraction of astrocytes seemingly arise from progenitors from the sub-ventricular zone (SVZ) (12) the question is if the evolutionary change in cytoarchitectural structure of the cortex, more specifically the outer sub-ventricular zone (oSVZ) (13), has any influence on human astrocytic development and heterogeneity, thereby making animal model translation more difficult. The astrocyte specific marker in rodent, GFAP, is for example also expressed by various other cell types in humans of which also the GFAPδ isotype mark specific progenitors not observed in mice (14). Human astrocyte heterogeneity is partly due to regional identity which has been mimicked *in vitro* (15, 16), whereas functional diversity seemingly is dependent on neuronal input (17-19).

Several current hiPSC derived astrocyte protocols (15, 20) assume that progenitor cells possess an intrinsic time course which transition differentiation potency. This means that sub-specific cells can be enriched from progenitor cells at specific temporal windows (4). If *in vitro* systems,

follow embryonic development it implies long and labor-intensive culturing. Alternatively, to shorten intrinsic developmental programs, and hence shorten model generation, one approach is to activate and drive developmental nodes to speed up the process, so called directed differentiation. However, a common problem of stem cell model generation is its immature phenotypes and fetal-like translatability. Despite this fact hiPSC derived models have demonstrated to be valuable in studying neural functionality (20) and disease mechanistic features (21) while also providing valuable information in phenotypic screening (22, 23). Human iPSC derived models show great potential to contribute to the development of new treatments for patients. Their possible application in the pharmaceutical industry are vast; providing large scale cultures, human genome origin, genomic diversity, functional evaluation, toxicity testing, ease of genomic editing for multiple downstream application as target identification, transcript overexpression, reporter systems, disease insertion and correction. Moreover, by utilizing established pharmaceutical infrastructure and previous results the information from hiPSC derived motor neuron models has shown to directly impact drug development by leading to the decision to launching a new clinical trial for treatment against amyotrophic lateral sclerosis (ALS) (24). Time will tell if the great potential of hiPSC derived models have a significant impact on drug discovery.

1.1 The Human Brain

The human brain contains approximately 100 billion neurons of which 20% reside in the neocortex, a structure associated with higher cognitive functions. To support the neuronal complexity, the human brain consists of the same amount or even higher number of glial cells. The neocortex is a hallmark in evolution existing as a unique structure observed among mammals. Its independent expansion along different evolutionary lineages highlight a positive selection for increasing neuronal numbers. A great enlargement is observed in primates, particularly humans, where the neocortex makes up 80% of the brain mass. In order to handle the great increase in neurons, without enlarging the skull, the neocortex expands its surface area by folding. Humans show a high level of gyrification as compared to the mouse which possess a smooth neocortex. Even though the layered structure of the neocortex is similar across mammals, its distribution is different, for example the human neuronal upper layers occupy a considerably larger part of the gray matter compared to rodents. This can be linked to the oSVZ populated by proliferative outer radial glia (oRG) which produce a high number of upper layer cortical neurons after gestation week (GW)17 (2, 25-27). Alongside the human cortical expansion follows the astroglia evolution which has undergone substantial changes. Astrocytes support and regulate the neuronal network. In addition to the development of primate specific astrocyte subtypes the cross-species astrocytes show diverse biology between mammals. Protoplasmic astrocytes, found in the gray matter, are 2.5 times larger in humans compared to rats. Additionally, human protoplasmic astrocytes occupy a 16.5 times larger volume, possess 10 times more primary processes and envelops approximately 2 million synapses compared to its rodent counterpart which envelope between 20 000-100 000 synapses (28). The intriguing question is if astrocytes contribute to increased cognitive function? Interestingly, when human glial progenitors were injected into the ventricle of newborn mice the progenitor population overtook the mouse brain outcompeting the host oligodendrocytes, protoplasmic and fibrous astrocytes. This resulted in improved performance on a variety of cognitive tests strongly indicating that human astrocytes improve cognitive ability in mice (29, 30)

Almost unthinkable, the structural complexity and neural diversity observed in the human brain all starts with lineage fate restriction of pluripotent stem cells which can generate any cell type in the body.

1.1.1 Neuroepithelial Stem Cells

Pluripotent stem cells of the inner cell mass develop upon gastrulation into the three different germ layers; endoderm, mesoderm and ectoderm. Further development of the ectoderm restricts cellular potency to become the primitive neuroectoderm forming the neural plate, consisting of a central part forming the definitive neuroectoderm which is bordered by the neural crest. During this time the definitive neuroectoderm forming the central nervous system (CNS) is being regionalized along the anterior-posterior (AP) axis defining regions that later become the forebrain (prosencephalon), midbrain (mesencephalon), hindbrain (rhombencephalon) and the spinal cord. The neural plate subsequently closes establishing the neural tube which buds off from the surrounding tissue. The mesendodermal tissue and notochord underlines the neural tube at the ventricle side while the non-neural ectoderm asides the dorsal parts. The notochord and non-neural ectoderm will affect the patterning along the dorsal-ventral (DV) axis, equivalent to the earlier mediolateral axis in the neural plate. Along the AP axis there is a wingless (WNT) signaling gradient that become caudally stronger until the initiation of the spinal cord where retinoic acid (RA) and fibroblast growth factor (FGF) gradients become increasingly more dominant. The DV axis has opposing gradients of sonic hedgehog (SHH), which is strongest at the floor plate and decrease dorsally and bone morphogenetic protein (BMP)s/WNTs, which are strongest at the roof plate and decrease ventrally. In addition to the mesendodermal tissue, notochord and non-neural ectoderm there are three major local signaling centers (secondary organizers): anterior neural ridge (ANR) at the anterior pole of the secondary prosencephalon, zona limitans intrathalamica (ZLI) at the anterior diencephalon and the isthmic organizer (ISO), at mid-hindbrain junction which refine signaling gradients and local identities. The three-dimensional grid (Figure 1) which patterns the neuroepithelial cells before neurogenesis onset at GW6-7 will set the rough positional identity which is the foundation of region development of the brain and its neural specialization (3-5, 31).

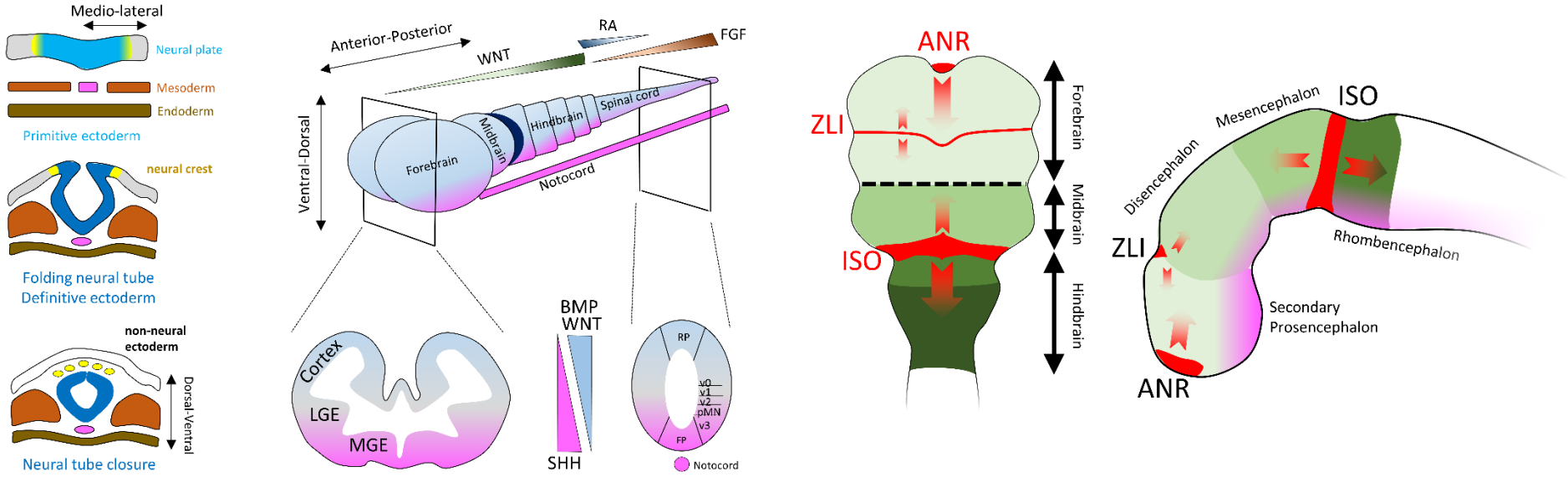

Figure 1 – Embryonic development of the brain is dominated by morphogenic gradients shaping a three-dimensional grid affecting cellular fate. Pluripotent stem cells in the inner cell mass undergo gastrulation of which the three germ layers are formed including ectoderm, mesoderm and endoderm. Following further specification, the neural plate is formed containing the primitive ectoderm which itself is defined by the developing definitive ectoderm, forming the CNS, and laterally flanking neural crest. The neural plate folds and bud off surrounding tissue forming the neural tube with non-neural ectoderm on the dorsal side while ventrally is the notochord and mesendodermal tissue. The surrounding tissue will affect the morphogenic gradients affecting neural patterning. Along the anterior-posterior axis there is an increasing WNT signaling gradient towards the spinal cord where RA and FGF gradients become more dominant. Governing the dorsal-ventral axis are two opposing gradients of BMP/WNT and SHH which is contributed by the non-neural ectoderm and notocord, respectively. Additionally, further refinements of the morphogen gradient space are secondary organizers ANR, at the anterior pole of the secondary prosencephalon, ZLI, at the anterior of the diencephalon, and ISO, at the midbrain-hindbrain junction. Adapted from (3-5).

1.1.2 Neurogenesis

Around GW7 during cortical development in the telencephalon neuroepithelial cells transform into radial glia (RG) in the ventricle zone and start to divide asymmetrically generating intermediate progenitors in addition to their symmetrical division expanding the progenitor population. At this stage in development there is a marginal and ventricle zone. As neurogenesis is initiated the cortical plate divides into the subplate and marginal zone which later develops into layer I. Radial glia scaffold keeps generating intermediate progenitors which terminally differentiates into post-mitotic neurons or undergo further rounds of cellular division before final differentiation. Early projection pyramidal neurons originating from ventricular RG (vRG) migrate along the radial fiber scaffold accumulating between the marginal zone and the subplate initially generating layer VI while later born neurons make up more superficial layers finishing at layer II. At GW9-12 the subplate reaches its maximum thickness and there is a gradual thickening of the cortex. Below the subplate, the intermediate zone, which later becomes the white matter, and the SVZ start to be distinguishable. In humans the SVZ give rise to two distinct layers; the iSVZ and, adjacent to it, the oSVZ defined by the inner and outer fiber. Neurons generated at GW13-15 occupy the middle layers of the cortex which at GW18 consists of six cortical layers. From this point to when neurogenesis ends there is an extensive production of neurons occupying the cortical layers in parallel with the increasing thickness of the SVZ. The ventricle zone is gradually reducing in size and by GW25-27 it has become one cell thick ependymal layer. Neuronal migration along the radial glia processes is complete at around GW28, at the same time, the deep and upper layers of the cortex have been generated. Nevertheless, tangential interneuron migration from the medial ganglionic eminence along the marginal and intermediate zone does not cease until the third trimester. By the end of the development, the cortical gray matter contains approximately 70-80% and 20-30% glutamatergic and GABAergic neurons respectively (2, 25-27). Notably, in human cortical development the ventricle radial glia scaffold, of which early newborn neurons migrate, is discontinued at GW17, losing its pial attachment and ending its processes in the oSVZ. This correlate to the change in progeny of oRG in the oSVZ which more frequently generates neurons in the upper layer of the cortex. Outer RG now make up the radial scaffold from the oSVZ to the pial surface. The time before and after GW17 is referred to as the continues and discontinuous scaffold stage, respectively (2) (Figure 2).

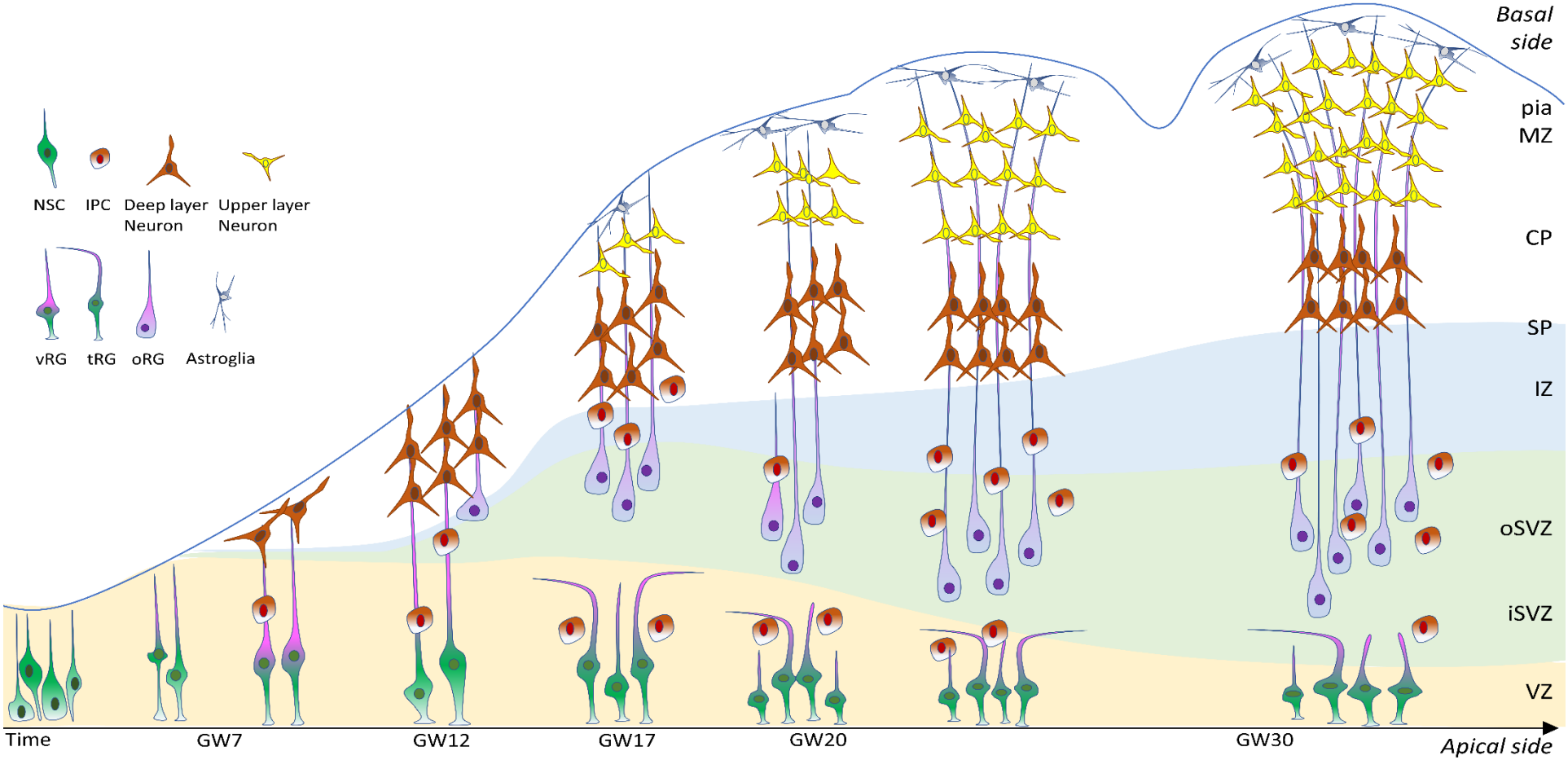

Figure 2 – Schematic representation of the embryonic development of the cortex. NSC stem cells develop into RG cells and start to divide asymmetrically generating IPC and neurons at around GW7. An initial thickening of the cortex happens around GW12 and subsequent generation of neurons start occupying the middle layers of the cortex which at GW18 structurally possesses all six cortical layers. Around this time in human development the radial fibers from the ventricle RG are discontinued no longer reaching the pia surface but which is maintained by the oRG stretching its fiber to the pia surface. The SVZ and cortex in parallel increase in thickness as oRG more frequently generate upper layer neurons. The neurogenesis and neuronal migration cease around GW30. NSC (Neural stem cells), IPC (intermediate progenitor cell), RG (Radial Glia), vRG (ventricular RG), tRG (truncated RG), oRG (outer RG), GW (gestation week), VZ (ventricular zone), iSVZ (inner subventricular zone), oSVZ (outer subventricular zone), IZ (intermediate zone), SP (subplate), CP (cortical plate), MZ (marginal zone). Adapted from (1, 2)

Development of different brain regions give rise to other major neuronal sub types. The mesencephalon divides along the DV axis to tectum and tegmentum containing the substantia nigra, an origin for dopaminergic neurons. Moreover, the DV axis of the rostral parts of the hindbrain, metencephalon, develop into the pons and cerebellum of which the raphe nuclei generating serotinergic neurons and locus coeruleus generating noradrenaline neurons originate from, respectively (31, 32) (Figure 3).

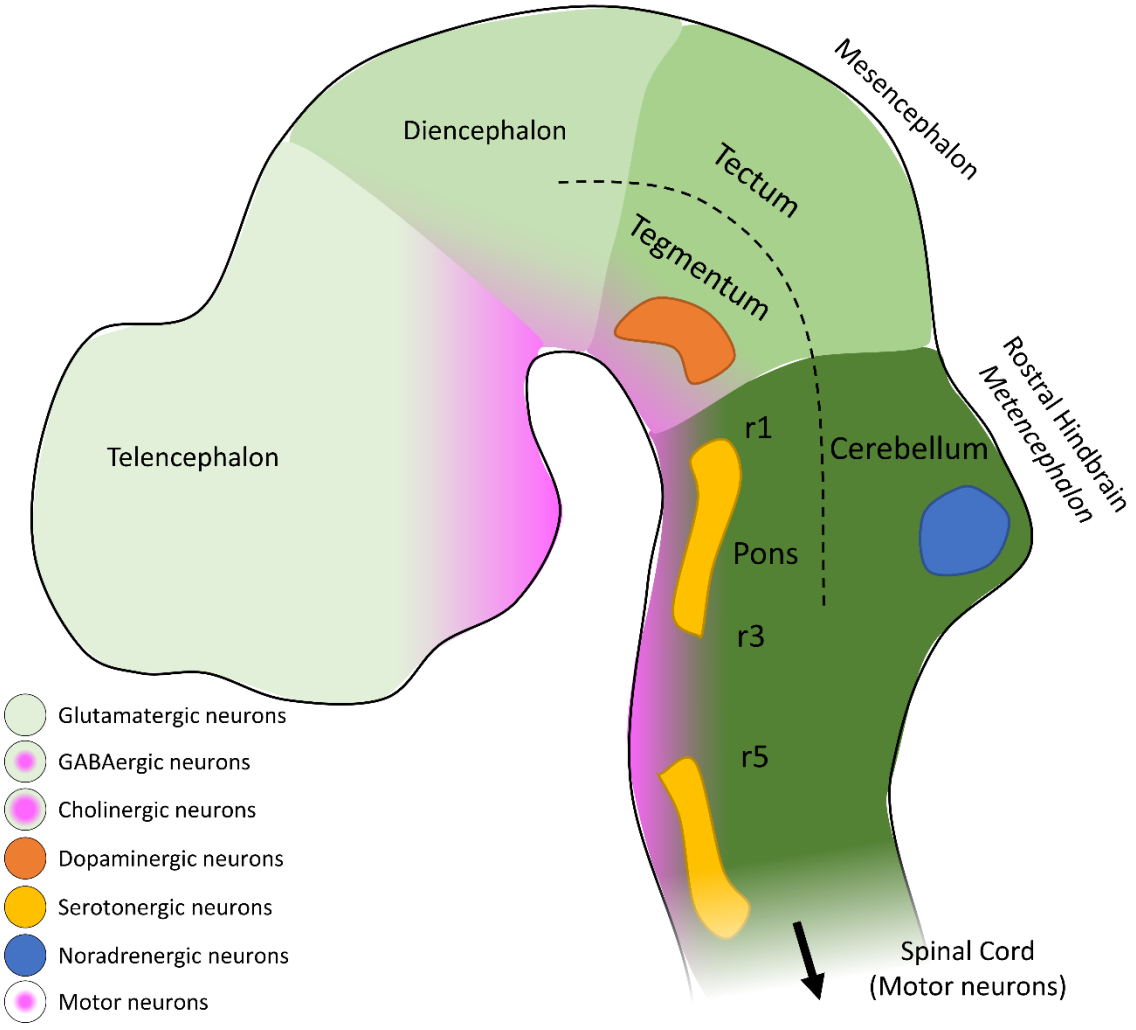

Figure 3 – Patterning of neural stem cells (NSC) affect the cellular regional identity contributing to the formation of different brain regions. Subsequently, the specific niche concentration of morphogens at precise areas of the brain will potentiate neural stem cells working as developmental origins for sub-specific neurons. Human PSC derived glutamatergic neurons are often generated from a patterning identity associated with the dorsal parts of the telencephalon which ventral domain harbor progenitors for GABAergic and cholinergic neurons. Dopaminergic neuronal progenitors arise in the ventral midbrain whereas serotonergic neurons develop from progenitors in the ventral part of the rostral hindbrain. Progenitors associate with an identity of the ventral part of the spinal cord can in turn give rise to motor neurons. Adapted from (32)

1.1.3 Gliogenesis

Gliogenesis is believed to be initiated around midgestation as neurogenic competence of progenitor cells switch into becoming gliogenic, which during development can be further divided into astrogenesis and oligodendrogenesis. As progenitors acquire an astrocytic fate they populate different regions of the brain. Two waves of astrocytic progenitors populate the pial surface of the cortex, at the time of the preplate formation and when the SVZ has formed. The SVZ and pial surface home progenitors which together migrate dorsally or descend into the cortical layers maturing as astrocytes. From the different progenitors in the SVZ another population of astrocyte occupy the white matter. Moreover, after neurogenesis is completed an additional source of transforming RG cells add to the astrocytic bulk of the brain which also expand in numbers by local proliferation. Regional identity and morphological characteristics are traditionally used to classify the heterogeneity of astrocytes. In relation to the evolutionary specific architectural structure of the human cortex and human astrocyte heterogeneity there is still a fundamental need to investigate the developmental programs of specific astrocytes (12, 33-36). In the developing spinal cord distinct homeodomains are linked to astrocytic progeny generating ventral fibrous astrocyte populations while dorsal region give rise to both protoplasmic and fibrous astrocytes. Similar regional specificity is shown for oligodendrocyte of the spinal cord originating from the pMN domain where motor neurons also originate (10). From animal studies oligodendrogenesis is viewed to occur in three distinct waves, both in the forebrain and the spinal cord. Originating from the medial ganglionic eminence (MGE) region in the forebrain a first wave of oligodendrocyte progenitor cells (OPC) is generated which through dorsal-tangentially migration occupy the forebrain. Through dorsal migration a second wave of OPCs populate the cortex of the forebrain which originated predominantly from the lateral ganglionic eminence (LGE). Lastly, the corpus callosum and overlaying cortex is infiltrated by progenitor cells from the dorsal parts of the SVZ and oSVZ which home the finalizing third wave of populating OPCs. This developmental dynamic in the human brain is however uncertain (11)

2 Modeling Embryonic Development and Cellular Function

A central problem of studying human neural cells is the limited access to human material. Isolation of primary cells is done primarily from fetal tissue, healthy tissue from surgical procedures and post-mortem samples. For post-mitotic cells, the limitation of material is pronounced since upscaling is not a possibility. Moreover, for mechanistic and functional studies, where genetic manipulation is desirable, transfection reagents often have a higher efficacy in proliferating cells. Purification of transfected post-mitotic cells without upscaling leave low number of successfully engineered cells. A solution is to use mitotic progenitors for which genetic manipulation, upscaling and banking is possible. However, extensive culturing of primary cells increases the risk of a phenotypic change thereby losing their high translational value.

In 2006, Yamanaka and colleagues could generate pluripotent stem cells by overexpressing four transcription factors in somatic cells (6). This provided an alternative to primary cells, and since reprogramming could be performed on cells collected using non-invasive procedures such as skin fibroblasts it removed ethical concern apparent with the use of embryonic stem cells. Induce pluripotent stem cells can theoretically develop into any cell type and is especially valuable in scientific fields where access to human primary tissue is limited, such as neuroscience. Human PSC can be scaled and banked, genetically manipulated and possess disease specific background. However, to generate cell types of interest specific differentiation protocols are needed which can direct and restrict cell potency to acquire the correct cell state.

Overexpression of OCT4, SOX2, MYC and KLF4 can reprogram mouse (6) and human fibroblast (37) into a pluripotent state. Reprogramming is also achieved with a different set of factors OCT4, SOX2, NANOG and LIN28 (38). To use hiPSC derived cells in clinical applications the use of non-integrating expression systems need to be applied to remove risks associated with genomic integration. There are several non-integrating reprogramming systems including RNA based sendai-virus (39), episomal DNA (40), RNA (41), protein (42) and small molecules, generating chemical induced pluripotent stem cells (43). Moreover, reprogramming (44) and hPSC cultures currently apply xeno-free systems (45, 46) removing previously necessary mouse feeder cells. Together this enabled development of good manufacturing practice (cGMP) compliant for pre-clinical and clinical applications (47). Moreover, it shows higher technical robustness and reproducibility. The strategy of overexpressing transcription factors for the acquisition of an alternative cell state also applies for the direct conversion of one somatic cell to another. This is for example achieved by converting fibroblast to neural progenitor cells (NPC) (48), neurons (49), and astrocytes (50).

The combination of primary cells, differentiation of pluripotent stem cells and direct reprogramming to desired cell types are all used to model embryonic development and cellular functions of the human brain (Figure 4).

Modeling human embryonic development and cellular function *in vitro*

The use of isolated, directed differentiated & reprogrammed cells

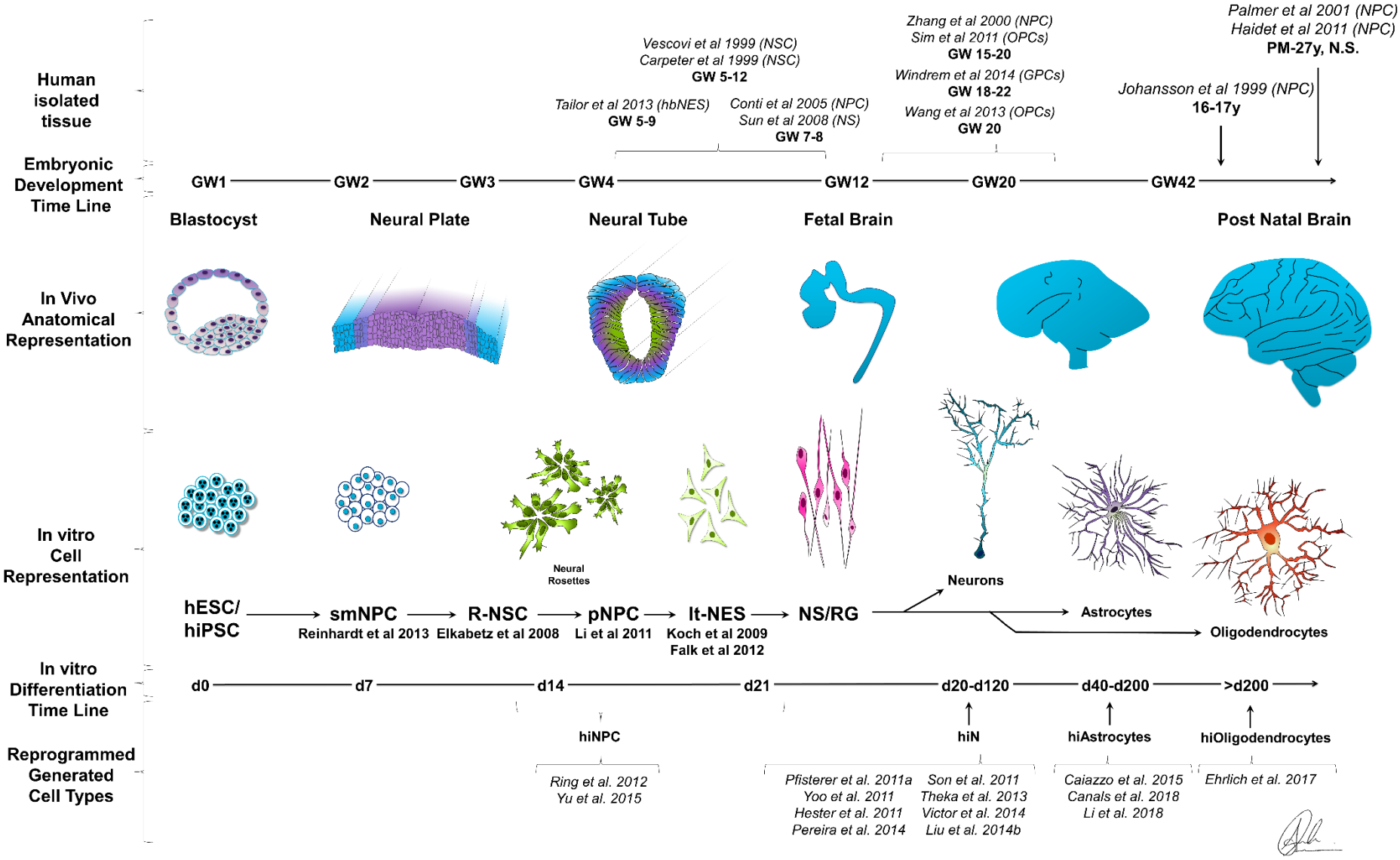

Figure 4 – Modeling human embryonic brain development and cellular function of neural cells with a human origin is performed by the isolation of primary cells, differentiation of pluripotent stem cells or the use of direct reprogramming. Accessing primary cells poses many challenges including ethical concerns and availability. Moreover, technical challenges of handling post-mitotic cells result in the focus towards isolating mitotic progenitors or stem cells. However, the research performed on primary cells provides valuable information and the possibility to gain knowledge about human cells *in vivo*. Isolation of primary progenitor cells is commonly performed from fetal tissue while multipotent stem cells have been successfully isolated from adult brain. The use of pluripotent stem cells requires differentiation protocols which commonly follow the embryonic development to generate cells of interest. Differentiation of hiPSC has capture several stages of ectodermal progenitor potency where smNPC can generate cells in both the PNS and CNS, while neuroepithelial stem cells R-NSC, pNPC and ltNES have been restricted to CNS. Further differentiation of neuroepithelial stem cells can generate RG and the three main cell types; neurons, astrocytes and oligodendrocytes. To generate more specified cells can take up to over 200 days *in vitro*, which in some aspect corroborate with *in vivo* development. In the aim to reduce the length of differentiation protocols researchers try and override developmental programs by either directed differentiation, speeding up the development process, or by overexpression of specific proteins which can directly reprogram one cell state to another. The later has been done by converting skin or pluripotent stem cells to neuroepithelial stem cells, neurons, astrocytes and oligodendrocytes. hESC (human embryonic stem cells), hiPSC (human induced pluripotent stem cells), smNPC (small molecule neural progenitor cells), R-NSC (Rosette neural stem cells), pNPC (primary neural progenitor cells), ltNES (long-term neuroepithelial stem cells), NS (neural stem cell), RG (radial glia), GW (gestation week), y (years of age), N.S. (not specified).

2.1.1 Neural Stem Cells

Development of more defined medium compositions establishing the N2 (51) and B27 (52) supplements have enabled better culture conditions for the development of neural cultures. Isolation of neural stem cells (NSC) was done in 1999 by Vescovi *et al.* who showed that isolating and propagating human fetal tissue from GW 6-12 as neurospheres require culturing condition using the combination of FGF and EGF (53). Addition of LIF also support NSC cultures but was shown to be redundant in NSC propagation (54, 55). Isolation of human fetal neural epithelial stem cells from embryo at GW5-7 can be maintained in long term culture with sustained tri-potent differential capacity (56). Deriving progenitor cells at GW8 show increased identify with RG populations of early brain development (57, 58).

Generation of human pluripotent derived NSC can be achieved by spontaneous differentiation. This occur during embryonic body (EB) formation in combination with FGF2 stimulation which upon plating generate rosette structures associated to neural tube formation (59). Even though propagation in culture conditions using FGF2 and EGF influence NSC to drift in cellular identity, Elkabetz *et al.* could maintain rosette NSC (R-NSC) phenotype for a few passages by isolating the inner cells of neural rosettes (60). The purity of long term human embryonic derived neural stem cells (lt-hESNSC) by mechanical isolation is crucial for long term cultures to avoid drifting in phenotypes (61). Establishment of hPSC derived long-term neuroepithelial like stem cells (ltNES) demonstrate that the cells can maintain tripotency and regional identity for over 100 passages (62). A directed approach using inhibitors of Activin/ transforming growth factor beta (TGFB)/ Nodal (63) or BMP pathway (64, 65) show to induce neuroectoderm formation from hPSC. Most efficient is the inhibition of both pathways simultaneously, called dual SMAD inhibition, applying Noggin and SB431542 to generate an enriched population of hPSC derived NSC (66). This can be further optimized with the use of small molecules like dorsomorphine (67, 68), DMH1 (69, 70) or LDN193189 (71). Additionally, isolation of NSC at an earlier state, primitive NSC (pNSC), is achieved by inducing WNT and janus kinases (JAK)/ signal transducer and activator of transcription proteins (STAT) signaling while maintaining dual SMAD inhibition. These cells lack polarization and they can generate rosette formations upon FGF2 stimulation (72). A common feature between R-NSC, ltNES, and pNSC is that they are restricted to CNS fate, limiting the derivation of motor neurons and neural crest cells. By using small molecules Reinhardt *et al.* managed to capture neuroepithelial cells, namely small molecule NPC (smNPC), which resemble cells of the neural plate borders having the potency to generate both motor and dopaminergic neurons (73). An alternative method is to directly reprogram fibroblasts generating induced NSC (iNSC) without a pluripotent intermediate state. Overexpression of SOX2 generates iNSC (48) which can be further improved by inhibiting Let-7 miRNAs resulting in upregulation of HMGA2 expression, affecting chromatin structure (74).

2.1.2 Neurons

Already in the 90's were neuroblasts isolated from fetus used in intracerebral transplantations for cell therapy of Parkinson's Disease (PD) (75). However, culturing and accessing sufficient

number of neuronal progenitors is challenging. This obstacle has been overcome by using hPSC derived dopaminergic progenitors supplying adequate cell numbers to treat primate PD models (76).

Since the establishment of the iPS-technology the number of established protocols for the generation of neuronal subtypes has increased greatly, demonstrating neurons derived from progenitors with fore-, mid-, hindbrain and spinal cord identities (68, 70, 77). Sub-specification is based on regionalization of neural progenitors, also called patterning. Physiologically patterning affects the neural plate and the neural tube formation, which further develop into the brain and spinal cord. Temporal and spatial morphogenic gradients along the AP and DV axis govern cellular specification. Morphogens patterning cues are mimicked *in vitro* during NSC generation to result in downstream sub-specification of neurons. Enrichment of regional specific progenitors is not only achieved by target progenitor stimulation but also via the restriction of neighboring progenitors. The latter is achieved via the use of opposing morphogens concentration, which results in a specified narrow regional milieu that affects the downstream neuronal sub-population purity.

2.1.2.1 Glutamatergic neurons

Initial NSC are consider to have a default cerebral cortical identity, also known as the activation-transformation model (3, 78, 79). Activation of neural tissue by induction would then acquire forebrain identity and caudalizing factors lead to a transformation to posterior neuroectoderm. This approach is used in the formation of hiPSC derived dorsal forebrain glutamatergic neurons (68, 80), even though a recent publication suggests a revision of current patterning model demonstrating that regional identity is acquired before neural identity (81). Dorsal forebrain PAX6/OTX2/FOXG1 expressing progenitors are generated upon ectoderm induction without stimulation of morphogens. Upon terminal differentiation toward glutamatergic neuronal fate the cells express TBR1 and SLC17A7 [VGLUT1] whilst acquiring identities of deep and upper-cortical layers (68, 80). However, generation of dorsal glutamatergic neurons is often accompanied with the generation of ventral GABAergic neurons (7, 82). Circumventing this problem and enhancing the purity of glutamatergic neurons is achieved by antagonizing ventral patterning using SHH inhibitors while keeping an anterior identity (7, 82).

2.1.2.2 GABAergic neurons

GABAergic neurons can be developed from the MGE and LGE of the forebrain generating GABA interneurons and striatal spiny GABA neurons, respectively. A NKX2-1 MGE forebrain identity is achieved by WNT signaling inhibition in combination with strong ventralization cues using high SHH (83). Differentiation of NKX2-1/FOXG1 progenitors can generate GABAergic neurons, but also CHAT+ cholinergic neurons derived from the same region (84). Technical optimization by limiting medium support for survival of cholinergic neurons can increase purity of GABAergic neurons up to 90% (83). Human PSC NKX2-1::GFP reporter lines also demonstrate a temporal effect of SHH stimulation on GABAergic

and cholinergic fractions. The temporal stimulation of SHH which generates the highest number of GABAergic neurons also display the most homogenous NKX2-1/FOXG1/OLIG2/ASCL1+ progenitor population (85). The importance of progenitor homogeneity is concurrent with other protocols, but optimal temporal stimulation is inconsistent (86). Further differentiation of NKX2-1/FOXG1/OLIG2 progenitors initiates expression of GABAergic interneuronal lineage markers ASCL1, DLX2, LHX6 and CALB1 (85, 86). Temporal discrepancies could be observed due to endogenous caudalizing effects as a result of the increased FGF19 expression. This phenomenon could be explained by early SHH treatment shifting regional identity towards caudal ganglionic eminence (CGE). Caudalizing effects can be counteracted with exogenous FGF8 stimulation maintaining anterior position or exaggerated with FGF19 resulting in calretinin (CALB2) interneuron subtypes of CGE (87). More mature interneuron markers somatostatin (SST), and parvalbumin (PVALB) can be detected in hPSC derived GABAergic neurons with MGE identity (85, 87). To generate a more dorsal identity of the LGE region, a balanced SHH stimulation is essential. Using an opposing morphogen to ventralization, activin A, can stimulate a LGE identity of GSX2, DLX2, BCL11B [CTIP2], and FOXP2 expression which differentiate to GABAergic neurons expressing striatal marker PPP1R1B [DARPP32]+ (88).

2.1.2.3 Cholinergic neurons

Many of the protocols generating GABAergic neurons from MGE progenitors also form a fraction of cholinergic neurons from the most ventrally patterned progenitors. Optimizing SHH temporal treatment can enrich cholinergic neuron derivation, resulting in a NKX2-1/FOXG1 population barely expressing OLIG2 (85). Basal forebrain progenitors differentiating to cholinergic neurons commonly express NKX2-1/FOXG1/LHX8 (89) followed by ISL1, CHAT and SLC18A3 [VAChT]. Long term EB expansion enriched basal progenitor population resulting in 90% pure cholinergic neuron cultures upon terminal differentiation (90).

2.1.2.4 Dopaminergic neurons

Dopaminergic neurons develop from ventral-midbrain progenitors. Human iPSC derived NSC are patterned by activation of the WNT and SHH pathways to regulate identity along the AP and DV axis, respectively (69, 71, 91-94). Fine tuning WNT signaling concentration gradient specify NSC to forebrain, midbrain or hindbrain fate which can be maintained during simultaneous DV specification using SHH signaling modulators. This methodology successfully generates midbrain progenitors efficiently differentiating into TH+ neurons (91). However, common progenitor markers of ventral mesencephalon (VM) LMX1A, FOXA2 and OTX2 poorly predicted dopaminergic neuronal sub-specification upon engraftment. Bioinformatic analysis of successful engraftments aligned with more caudal progenitor identity (77). Temporal addition of FGF8b, after VM patterning, could generate caudal VM, expressing EN1, SPRY1, PAX8, CNPY1, and ETV5, markers demonstrating good prediction of high dopaminergic sub-specification outcome (77). Technical optimization including the use of human recombinant laminin 111 and switch in basal medium composition resulted in a protocol

generating 40 fold more FOXA2+/LMX1A/B+ progenitors expressing EN1/OTX2/LMX1A (77).

2.1.2.5 Serotonergic neurons

Ventral hindbrain progenitors give rise to serotonin neurons. Progenitors from the rostral and caudal hindbrain differentiate into median raphe serotonin neurons and spinal cord connecting serotonin neurons, respectively (32). Regulation of the AP WNT gradient can derive hindbrain progenitors expressing caudal and spinal cord identity markers (70). Specific CHIR99021 concentration generate rostral hindbrain HOXA2+ progenitors acquiring a ventral NKX2-2/NKX6-1 identity during simultaneous SHH stimulation (70). Serotonin specification, indicated by high homogeneity of GATA2, need temporal addition of FGF4 for the acquisition of FOXA2 serotonergic fate. Terminal differentiation can generate 60% serotonin neurons assessed by TPH2, GATA2 and SLC18A2 [VMAT2] expression and the functional release of serotonin upon depolarization (70).

2.1.2.6 Motor neurons

Spinal cord progenitors are able to generate motor neurons originating from ventral-dorsal OLIG2+ region. Using RA and SHH can direct the differentiation toward OLIG2+ progenitors which can develop into ISL1 and CHAT positive motor neurons (95, 96). High SHH concentration generate progenitors with NKX2-2 regionality. To achieve more dorsal regionality WNT activation can antagonize ventral induction generating almost pure OLIG2+ populations. Terminal differentiation generate MNX1, ISL1 and over 90% CHAT+ motor neurons (97). Furthermore, Qu *et al.* achieved higher viability of motor neurons by exchanging Matrigel to a defined mixture of laminin, fibronectin, collagen I and collagen IV (98).

2.1.2.7 iNeurons

Another approach to generate sub-specific neurons is the direct conversion without going via stem cell state. Direct reprogramming into human induce neurons (hiN) is possible by transduction ASCL1, POU3F2_v1 [Brn2a] and MYT1L (49). The application of small molecules including dual SMAD and WNT activation lead to a significantly increased efficacy. This method generates mostly GABAergic neurons, however, adding LMX1A, LMX1B, FOXA2 and OTX2 to the vector mix provide the possibility to generate dopaminergic neurons (99). Generation of dopaminergic iN from hiPSC is achieved by using ASCL1, NR4A2, and LMX1A (100). Direct conversion of human fibroblast into hiN is performed by using mircoRNAs miR-9-3/5p [miR9/9*] and miR-124 (101). Increased efficacy to produce iN with GABAergic striatal medium spiny nature can be obtained by transducing BCL11B [CTIP2], DLX1, DLX2, and MYT1L together with miR-9-3/5p and miR124 (102). Interestingly, regulating microRNA circuits by shRNA inhibition of the polypyrimidine-tract-binding (PTB) protein can drive neuronal transdifferentiation from rat (103) and human fibroblasts (104). Generation of other subtype specific neurons such as iMN from human fibroblast and hESC can be achieved using a 8 factor combination (ASCL1, POU3F2, MYT1L, LHX3, MNX1

[Hb9], ISL1 and NEUROG2) (105) and 3 factor combination (NEUROG2, ISL1, LHX3), respectively (106).

With the combination of established ectoderm differentiation protocols and the modulation of morphogenic gradients hPSC serve as a model of early embryonic brain development by acquiring different regional identities. This, in turn can reflect cellular transition of fate restriction and differentiation potency as specific progenitors acquire sub-specific neuronal phenotypes. In addition, genetic techniques introducing inducible neurons provide an alternative and effective approach of generating sub-specific neurons (Figure 4).

2.1.3 Astrocytes

Astrocytes are important for both brain homeostasis and functions. Moreover, astrocytes in non-human primates and humans are highly diversified across evolution to plausibly match the needs of neuronal subtypes. However, human cellular models of astrocyte biology have not developed equally to neurogenic models. This might be due to the lack of consensus on how to define an astrocyte. The next section will review astrocyte biology and the development of human astrocytic *in vitro* models.

3 Astrocyte Biology

The human brain consists of various specialized neural cell types (107), which during evolution co-developed to meet the needs of one another (108). Astrogenesis takes place after neurogenesis where the main astrocyte subtypes can be specified into gray and white matter astrocytes, also known as protoplasmic and fibrous astrocytes, respectively (36). Astrocytes at the pial surface, layer-1 astrocytes, show similar morphology and GFAP expression as fibrous astrocytes (36). Populations of astrocytes also exists in the spinal cord where progenitor homeodomain regulate spatial location of gray and white matter astrocytes (109, 110). Müller cells in the retina, Bergman glia and protoplasmic velate astrocytes in the cerebellum are yet another example of glia diversity (111). There are also astrocytes specific to primates and humans including interlaminar and varicose projection astrocytes (36, 112-114). In addition, characterized by their GFAP and glycogen granules, adult astrocytes in the SVZ, namely type-B1 and -B2 cells, are a tripotent stem cell population having the potential to generate neurons, astrocytes and oligodendrocytes (115). The astrocytic functions are diverse, and the list of functions associated with astrocyte biology has been expanding over the last decades. Astrocytes provide structural and metabolic support together with blood brain barrier (BBB) functions, regulating cerebral blood flow, synaptic homeostasis, inflammatory response to injury, and neuronal circuit integration, an area which has gained more attention the recent years. Astrocytes influence synaptogenesis and the synaptic connectivity, most likely also being part of fine tuning neuronal circuits. There are several excellent reviews on astrocyte biology (10, 12, 113, 116-120)

3.1 Astrocytes in Early Brain Development

Regional and morphological differences display astrocyte heterogeneity in adult brain even though GFAP expression is common across sub-types (112, 121). Additionally, differential gene expressional along the DV and AP axis in adult mice (122) indicate regional specific developmental origins and contribution of intracellular programs to astrocyte heterogeneity. However, neuronal niche dependent effects on astrocyte heterogeneity is also observed (19, 123). The relative contribution between intracellular developmental programs and extrinsic signals to astrocyte heterogeneity remains to be fully determined.

The generation of astrocytes during early brain development are believed to arise from RG, progenitors of the SVZ, local cellular division, and CSPG4 [NG2] (from here on named NG2), progenitors (12). During early development of the human cortex as the formation of the preplate occur the first wave of layer-1 astrocytes derived from ventricular zone (VZ) progenitors move to the subpial glial limiting membrane. As neural progenitors transition from being neurogenic to gliogenic after the formation of the gray matter a second wave of astrocyte from the SVZ migrate and populate the pial surface (124, 125). In adult human cortex the pial surface and adjacent regions are occupied by three subtypes of astrocytes; pia/layer-1 fibrous, interlaminar and protoplasmic astrocytes (112). Notably, protoplasmic astrocyte predominantly does not express GFAP but glutamate transporter SLC1A2 [EAAT2, GLT1] and SLC1A3 [EAAT1, GLAST] (126). The progenitor identity of pia/layer-1 and protoplasmic astrocytes

are shown in mice to distinctly differ from one another (33). Since the interlaminar astrocyte is not observed in rodents it is not known if this sub-type arises from a progenitor different from pia/layer1 and protoplasmic astrocytes.

The astrocytic SVZ progenitors migrate and populate all six cortical layers but also remain in SVZ and white matter (12). For the generation of protoplasmic and fibrous astrocytes there are indications that the two sub-types are derived from different progenitors in the SVZ (33). Genetic deletion of *OLIG2* affect the formation of fibrous but not protoplasmic mouse astrocytes (127). In contrast, overexpression of *OLIG2* increases glia production of astrocytes and oligodendrocytes (128) indicating *OLIG2* to be an important intrinsic fate determinant for SVZ progenitor derived astrocytes of the OLIG2 niche in the MGE region. Additionally, early studies from human fetus indicate that RG type II with only ventricle attachment (124), similar to tRG definitions (2), develop into fibrous astrocytes, while RG type I develop into protoplasmic astrocyte after gray matter formation (124). At the end of radial neuronal migration RG transform by retracting their ventricle attachment moving the soma from the VZ towards the intermediate zone (IZ) transitioning into an astrocytic fate (125). The development of protoplasmic astrocytes is suggested to progress via a dorsal migration to the pial membrane and later descending into the cortical layers acquiring a maturing phenotype (33, 125, 126). Astrocyte numbers are further increased by local division to expand the protoplasmic astrocytes population in the cortical gray area (129). NG2 glia population, commonly associated as OPCs, have the potential to generate astrocytes. Lineage tracing of NG2 cells indicate in contrast to the possible contribution to the ventral forebrain astrocyte population that NG2 progenitors do not substantially contribute to the astrocyte population in the dorsal cortex (130, 131). Isolation of human glial progenitor cells (GPC) identified by NG2 expression in transplanted mice do show astrocytic differentiation competence (29, 30). However, generation of astrocytes from NG2 cells in physiological and *in vitro* system is still controversial.

Astrocyte associated cell types in the hindbrain originating cerebellar structure include Bergman glia and protoplasmic velate astrocytes (111). These two subtypes share a common developmental progenitor (111). Interestingly, the molecular diversity between these cell types is shown to be dependent on neuronal input of SHH signaling. Inducing SHH in velate astrocyte made them become more Bergman like astrocytes (17).

Progenitor origin and astrocyte heterogeneity in the spinal cord follow a more strict DV regional patterning. In the spinal cord, progenitor domains p1, p2, and p3 generate ventral white matter astrocytes VA1/VA2/VA3, respectively, while the pMN OLIG2-domain in between p2 and p3 generate oligodendrocytes (109). The p0 DBX1-domain generate both protoplasmic and fibrous astrocytes which can also be observed for the overlaying dP1-dP6 PAX3-domains which gives rise to all dorsal astrocytes (110). The dynamics in generating gray and white matter astrocytes in the spinal cord is linked to ASCL1 expression where KO of *ASCL1* increase the number of gray matter astrocytes in relation to a reduction in both white matter astrocytes and oligodendrocytes (132). Long-term studies indicate that the distribution of astrocytes in the spinal cord are determined during embryogenesis, and that regional specific

astrocytes tend to only support neurons associated to its own region compared to adjacent regions (110). The ventral and dorsal region-specific transcription have direct physiological impact where ventral expression of SEMA3A is critical for sensorimotor circuit integrity (133).

Even though studies of astrocyte generation and developmental origin in non-primate models provide very valuable information, astrocyte biology of humans show clear differences compared to non-primate models. Beside pial layer 1 astrocytes and protoplasmic astrocytes there is an additional subtype exists at the pial surface of higher primates not observed in rodent, the so-called interlaminar astrocytes. Interlaminar GFAP/CD44+ astrocytes extend poorly branched processes into the subcortical layers ending on blood vessels (36). Additionally, there is a second primate-specific subtype mainly situated in layer 5-6 called varicose projection astrocyte which also show GFAP/CD44+ expression. (36, 112). The evolutionary expansion of the cortex in higher primates is highly associated with the expanding progenitor population of the oSVZ, which contribute to the formation of the upper layers of the cortex (2, 13). How the progenitors of the SVZ contribute to the astrocyte heterogeneity and if this evolutionary diverged zone harbor progenitors responsible for human astrocytic subtypes is still unclear (Figure 5).

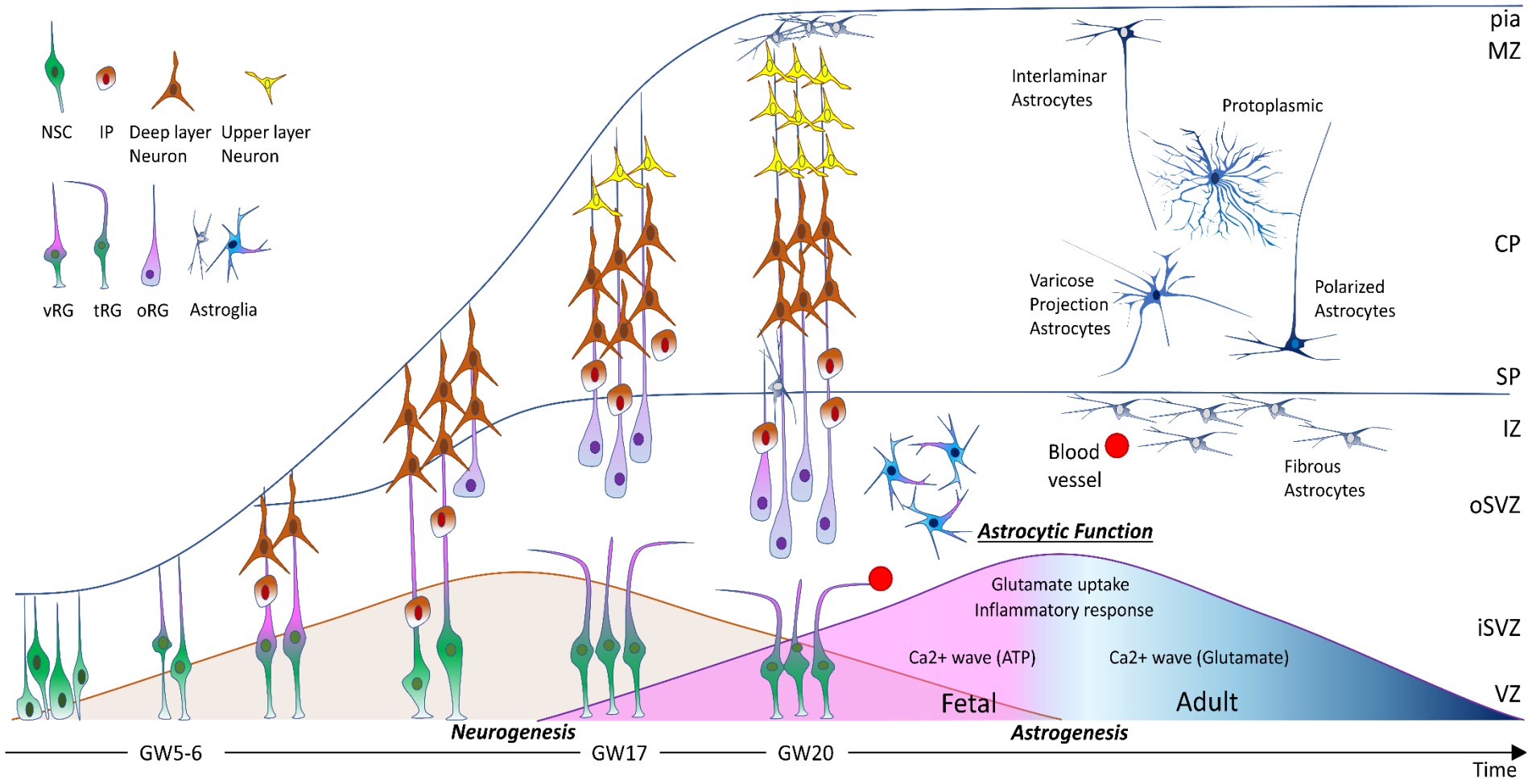

Figure 5 – Representation of the embryonic development of the neocortex highlighting the transition of neurogenesis to astrogenesis. Neurogenesis is initiated around GW7 of which vRG derived neurons occupying the deep layers of the cortex. Expansion of the oSVZ occurs with the transition of vRG to tRG and oRG at GW17-18. Upper layers of the cortex are then expanded by the contribution of oRG derived neurons. Neuronal production ends after mid-gestation in relation to the initiation of gliogenesis and the generation of astrocytes. However, there is gap in the understanding of how progenitor composition contributes to the heterogeneity in human astrocyte biology and its functional acquisition. NSC (Neuroepithelial stem cells), IP (intermediate progenitor), RG (Radial Glia), vRG (ventricular RG), tRG (truncated RG), oRG (outer RG), GW (gestation week), VZ (ventricular zone), iSVZ (inner subventricular zone), oSVZ (outer subventricular zone), IZ (intermediate zone), SP (subplate), CP (cortical plate), MZ (marginal zone). Adapted from (1)

3.2 Non-coding Transcripts in Neural Biology

The rapid development of sequencing techniques and computational power have made transcriptomic profiling more accessible with increasing granularity. This has demonstrated that non-coding RNAs have a bigger role in genome regulation than previously anticipated, even though we are just in the beginning of understanding its full regulatory contribution.

Non-coding RNAs are divided up in long and short RNAs based on if the transcript is longer or shorter than 200nt, respectively. Further classification is possible, but exclusive guidelines are lacking (134). Evolutionary trends point towards a correlation between increased developmental complexity and increased non-coding repertoires (135), some being exclusive for primates (136) and enriched in the CNS (137). Protein-coding genes have in general higher expression than long non-coding RNAs (lncRNAs) which, however, display higher tissue specificity (138). Long ncRNAs can regulate and maintain pluripotency in PSC (139, 140) as well as developmental potency in neural stem cells (140) subsequently affecting and defining differentiation outcome (141) and functional development (142). Another group of ncRNAs called microRNAs (miRNAs), observed as small RNAs of ~18-25nt in length regulate a vast variety of cellular processes (101, 144, 145). MicroRNAs can target and regulate translation or function of other RNAs in a spatiotemporal manner at either the cell level or specific subcellular locations, which is important in highly polarized cells (143). Neural differentiation is highly linked to miRNA expression which induce neuronal fate directly (101) and is an important player in the neurogenic-to-gliogenic switch in NSC (144). Additionally, neuronal communication via miR-124-3p can regulate translation of astrocyte specific glutamate transporters in distal processes (145), shown to possess subcellular protein synthesis (146), regulating functional state. The interaction between lncRNAs and miRNAs has a role in neurodevelopment and in a primate specific context (147). The lncRNA for neurodevelopment (lncND), specifically expressed in certain primates is targeted at 16 sites by miR-143-3p. Downregulation of lncND or overexpression of miR-143-3p results in decreased proliferation and increased neuronal differentiation, respectively (147). miR-143-3p regulates notch signaling, which plays an important part in regulating progenitor proliferation and it is associated to primate neocortex expansion (148). Gain of function of lncND can expand the RG population in the mouse cortex (147) demonstrating the interplay between lncRNA and miRNA in the governance of cellular state.

3.3 Astrocyte Characterization

Distinguishing between different cell populations is most commonly performed by studying transcript and protein expression. However, so far this approach has been challenging when defining astrocyte population since there are no unique genes identified that are only expressed by astrocytes. Additionally, the highly associated astrocytic marker GFAP, predominantly expressed by astrocytes in rodents, stain RG cells and adult stem cells in humans, demonstrating species variations in marker expression. Furthermore, GFAP mRNA expression in glia cells is not always associated with protein expression (149). Protein and RNA expression can be complemented by morphological characterization when studying astrocytes *in vivo*, but is not often conclusive *in vitro*. Therefore, astrocyte characterization needs to include several features to better define its cell type and subpopulations including morphology, combination of gene and protein expressions together with functional properties.

3.3.1 Astrocyte Morphology

The morphological and regional identity can define the major subclasses of astrocytes. Proteoplasmic astrocytes located in the cortical regions have largely branched tertiary processes enwrapping blood vessels while fibrous astrocyte in the white matter display more long unbranched processes. Layer 1 pial astrocytes have a fibrous morphology lining the pial matter. In higher primates there are two additional astrocyte sub-types; interlaminar astrocytes located in layer 1 of the cortex branching down into cortical layers 2-4 terminating their endfeet on blood vessels, and varicose projection astrocytes located in layers 5-6, which given by its name extend long projections across brain layers (112).

GFAP is one of the most commonly used markers for visualizing astrocytes. However, GFAP staining only partially displays the astrocytes morphology which has numerous fine peripheral astrocyte processes (PAPs) generating a more cloud looking morphology, rather than the classical star shape, giving astrocytes their name. These fine processes can be as thin as 30-50nm (150). Diastolic labeled GFAP positive cells show that GFAP staining only display roughly 15% of the cell volume, (35, 151), where PAPs roughly define up to 80% of an astrocytes surface area (152). Astrocytes connects to smaller vessels and capillaries, which in contrast to connections to larger vessels, visualized by GFAP staining, can be identified with intense AQP4 staining (153). Additionally, by only staining for GFAP the major protoplasmic astrocyte population is not visualized since it does not express GFAP (36, 126).

Besides the fact that humans display astrocyte subpopulation not present in mice there is a significant difference in astrocyte morphology between human and mouse. Human protoplasmic astrocytes display 2.6 times larger diameter and extend 10 times more primary processes than their mouse counterpart. A larger size can also be observed regarding human fibrous astrocytes compared to the mice (112). The increased territory of human astrocytes compared to mice astrocytes has also been shown *in vitro* (154). A single astrocyte domain covers up to 2 000 000 synapses compared to the mouse astrocytes which covers around 120 000 synapses (112). Interestingly, even though protoplasmic astrocytes are connected there is

very low overlap between cellular domains. Notably, in contrast to neighboring protoplasmic astrocytes is the domain border of a protoplasmic astrocyte not respected by CD44+ branches from interlaminar and varicose projection astrocytes which can cross and intrude protoplasmic domains (36).

The morphology of isolated primary astrocyte is affected by *in vitro* culturing, which been demonstrated to be dependent on the application of fetal bovine serum (FBS). Defined medium or FBS containing conditions show significant differences on morphology, maintaining profound stellate morphology while inducing polygonal morphology, respectively (154, 155). Protoplasmic astrocytes *in vivo* can lose their bushy morphology acquiring a reactive phenotype reducing SLC1A2 while increasing CD44 expression (121). Since *in vitro* culturing can induce naturally occurring transformation of protoplasmic astrocytes it is important that *in vitro* culture can sustain a non-reactive environment, especially when studying the stellate protoplasmic astrocyte biology which is linked to the generation of the tripartite synapse (156).

3.3.2 Astrocyte Transcriptional Profiling

Transcriptomic profiling has undergone a big technical development in recent years with increased granularity by single cell transcriptome profiling. This provided valuable information about the cellular architecture of the brain highlighting the variety of neural cell specifications (107). There are several transcriptomic databases classifying astrocyte associated transcripts in non-pathological (154, 157-159) and pathological conditions (160-162). In addition to bulk sequencing some single cell studies capture astrocyte characterization (163-165). However, interpreting transcriptomic profiles should be done in the light of the cellular background of the transcriptomic samples. Human astrocytes isolated under defined conditions show an inflammatory induced response upon FBS culturing and similarities to glioma cells (154). Using inflammatory categorization (166), Zhang *et al.* demonstrate that serum-free isolated astrocytes show low association to a reactive astrocytic profile in contrast to astrocytes exposed to serum (154). Several astrocytic transcriptome profiles are based on samples isolated with the presence of serum (157, 160-162, 167, 168).

Rodent models are commonly used for studying astrocyte biology for which several astrocyte enriched transcripts overlap with human astrocytes; *GFAP*, *ALDH1L1*, *AQP4*, *CLU*, *SLC1A2*, *SLC1A3, SLC4A4, ELOVL2, ACSBG1, TTYH1, ATP1B2, SOX9* (154). However, comparing human fetal (GW17-20), juvenile (8-18yrs) and adult astrocytes (21-63yrs) with mouse astrocytes identify human specific astrocyte enriched genes in addition to transcripts associated with adult mature astrocytes; *FAM198B, RYR3, AMY2B, LRRC3B, GPR98, CPE* (154). Single cell analysis of fetal and adult human brain tissue validated previously identified astrocyte associated genes, but neither study displayed any astrocyte sub-type specification (165). Since human brain expansion is associated to the expanding SVZ during embryonic development, several studies focus on characterizing progenitor populations between GW12-23 including RG, vRG, oRG (148, 165, 169-174). The question is if the heterogeneity of the SVZ progenitor population is associated with later developing sub-classes of astrocytes? Transcriptomic identity of oRG cells show overlap with astrocyte associated transcripts (148). The transition

of transcriptomic profiles from vRG/fetal astrocytes, oRG to mature astrocyte is studied in HEPACAM isolated cells from cerebral cortical spheroid models (175), which indicate the possibility of cell identity development of RG to astrocytes. Further development of single cell RNA sequencing analysis offers a time derivative of gene expression, providing rate and direction of entire transcriptomes, which enables further stratification of cellular transition. The analysis methodology demonstrates a developmental node between RG and astrocytes in rodent hippocampus associated to *HES1* expression followed by *AQP4* cellular expression (176).

Non-coding RNAs can be cell type specific where certain miRNAs are enriched in cerebral and cerebellar astrocytes (167), but also associate to anatomic location and age in human astrocytes (177). Long non-coding RNA show similar characteristics where certain lncRNAs define RG (165, 171) and astrocyte populations (164).

3.3.3 Astrocytic Protein Expression

Astrocyte protein expression together with morphological and anatomic location is the basis for classical astrocyte sub-type specification. Layer-1 pial and interlaminar astrocytes together with fibrous and varicose projection astrocytes all are positive for GFAP/CD44 staining, which is predominantly not expressed by protoplasmic astrocytes (36, 112, 126). Intracortical astrocytes with similar morphology as protoplasmic astrocyte have been identified to stain for GFAP/CD44. However, since the cell characteristics are not present during fetal development and show high variability in adult samples, it might be an acquired phenotype which the author hypothesises to be linked to pathological change (36) as increased GFAP expression is associated with age (178). Protoplasmic astrocytes in adult brain express SLC1A2 and SLC1A3 which are also observed for pial and interlaminar astrocytes during development (126), but GFAP/CD44+ astrocytes generally show low expression of SLC1A2/3 (36, 126). Staining for AQP4 visualizes smaller vessels and capillaries (153) as it is expressed in the astrocytic endfeet of protoplasmic astrocytes (36). However, for CD44+ interlaminar and fibrous astrocytes AQP4, is observed around the soma and along processes instead of concentrating around blood vessels (36). S100B expression is predominantly expressed by protoplasmic astrocytes in the cortex but is expressed by GFAP+ and additional cell types in other parts of the brain (18, 179). GFAP expression is detected around GW13 in humans, defining a RG population. GFAP expression is also detected in stem cell populations in specific regions of the adult human brain including rostral migratory stream (RMS), the olfactory bulb (OB) and expression along the ventricle in the SVZ (180, 181).

Besides being important during glial developmental onset (16, 182, 183) SOX9 has been shown to be a reliable and exclusive marker for astrocytic identity in the adult brain, except for ependymal cells and neural progenitors in neurogenic regions (184). Other proteins related to astrocytic biological processes include key enzyme in the foliate metabolism, ALDH1L1 (185), connexins GJB6 [Cx30] and GJA1 [CX43] (186, 187), potassium homeostasis, KIR4.1 (188), vitamin A metabolism, ALDH1A1 (189), glutamine synthesis, GLUL [GS] (190, 191), and thyroid hormone receptors SLC16A2 [MCT7/8] and SLCO1C1 (192).

Besides detection of protein expression can the fluorescent dye sulforhodamine 101 (SR101) be used to stain astrocytes *in vivo* (193). However, SR101 labels both astrocytes and myelinating oligodendrocytes since the dye is transferred via existing gap junctions between astrocytes and oligodendrocyte (194). Recently it was demonstrated that SR101 is taken up via the thyroid hormone receptor SLCO1C1 (192), which together with SLC16A2 [MCT7/8], are the major transporters for T4 (195, 196). Via T4 conversion by DIO2 activity, astrocytes serves as a major source of neuronal T3 during fetal development, which in adults is accessible directly via circulation (196).

3.3.4 Neuronal Effect on Astrocyte Heterogeneity

Recent technical development of single cell RNA sequencing has revealed a large subset of neuronal transcriptional identities (107, 197). However, the same technique has not diversified the astrocyte population to the same extent. One part of the explanation could be that astrocytes require a spatial and temporal plasticity of cellular specification and function which is later regulated by the local milieu and neuronal input. Peripheral astrocyte processes contain both ribosomes and rough ER translating local mRNAs providing the possibility for local maturation of membrane proteins. This subcellular architecture enables functional polarization of astrocytes (146). Secretion of neuronal exosomes containing miR-124-3p can induce translation of glutamate transporter SLC1A2 in astrocytes (145). Furthermore, *in vivo* experiments show that neuronal expression of SHH in the cerebellum affects gene expression in both adult mice and during development of Bergmann glial (BG) and velate astrocytes (VA). Increased SHH signaling in VA induced transformation to become more BG-like. SHH regulation also changes the electrophysiological properties of BG (17).

Astrocyte heterogeneity can be associated with its position in the upper or deep cortical layers (123), which cannot be explained by diversified progenitor populations since clonal analysis indicating a common progenitor (33). Interestingly, the cortical heterogeneity disappears in mice lacking laminar structure (123). This indicates that layer-specific properties of astrocytes are not due to intrinsic developmental programs, but as an effect of extrinsic factors associated to neuronal layered structures (123). Modeling RG development in neural columns can show that neuronal cortical placement is followed by protoplasmic astrogenesis, where astrocyte precursors migrate radially distributing across the cortical layers before final rounds of division (18). This enables the cortical layers to associate diversity and regional specification of astrocytes derived from the same RG precursor. Additionally, maturation of astroglia is influenced by local neuronal signaling. Astrocytic domain development and expression of the glutamate transporter, SLC1A2, is significantly reduced by silencing glutamatergic synaptic activity in SLC17A7 [VGLUT1] KO mice (19). Similar arborization of astrocytic process and lowered SLC1A2 expression is observed when silencing the metabotropic receptor GRM5 [MGLUR5] which mediate glutamatergic signaling in astrocytes. This effect is not observed in hypothalamus, where glutamatergic SLC17A7 signaling is not dominant. Together this indicates that functional maturation of cortical astrocytes is regulated by glutamatergic

signaling from local neurons (19). Moreover, neuronal effect on astrocyte function is also observed *in vitro,* significantly impacting astrocytic glutamate uptake (198).

3.3.5 Astrocyte Functionality

Astrocyte functionality is heterogenous and even though the contribution to brain function is not fully understood numerous key features associates to astrocyte biology. There are several excellent reviews highlighting the diversity of astrocytic function including glutamine-glutamate/GABA cycle (199, 200), gliotransmission (201), calcium signaling (202, 203), potassium homeostasis (204), energy storage and support (205, 206), astrogliosis (207-210), water homeostasis (211), astrocyte-neuron lactate shuttle (212), thyroid metabolism (196), synaptogenesis (213), regulation of synaptic behavior and neurotransmission (214-216) and BBB interactions (217). Evaluation of glutamate uptake, inflammatory responsiveness, and calcium signaling are commonly performed in hPSC derived astrocytic models and is used as a measurement of astrocytic identity.

3.3.5.1 Astrocyte Association to Glutamate/GABA Cycle

Glutamate and GABA are secreted from the presynapse by glutamatergic and GABAergic neurons mediating excitatory and inhibitory signals, respectively. The neurotransmitters are taken up by postsynaptic neurons to continue neurotransmission. Depolarization and release of glutamate increase the concentration of glutamate in the synaptic cleft to the magnitude of 100µM-1mM. For repeated transmission the concentration of glutamate in the synaptic cleft needs to be lowered reaching resting concentrations around 1-10µM (218, 219). Lowering the glutamate concentration is a key function for astrocytes which express glutamate transporters SLC1A3 and SLC1A2 (126) being responsible for the majority of glutamate transport (220), appreciably by SLC1A2 (221). A decrease in synaptic concentration occurs within 1 millisecond (218) via rapid transport of SLC1A2/3, a rate which increasing during development in concordance with increased SLC1A2 expression (221). Failure to remove excess glutamate in the synaptic cleft can lead to excitotoxic effects and damaging of neurons (222). Glutamate transporters SLC1A2/3 are sodium dependent internalizing 3 Na^+ per glutamate molecule which needs to be exported out from the cell by Na^+,K^+-ATPase to restore ion balance. The Na^+,K^+-ATPase ATP1B2 and ATP1A2 are associated to astrocytes (154) and RG (148, 165), respectively. Glutamate uptake is an energy demanding process. This is balanced by oxidative metabolism of glutamate generating α-ketoglutarate entering the TCA cycle via transamination or energy producing reactions governed by BCAT2/ GPT [ALAT] and GLUD1 [GDH], respectively (223). Exogenous glutamate is suggested to be primarily metabolized by GLUD1 (224), which is highly transcribed in astrocytes (225). By GLUL [GS] glutamate is also converted into glutamine, an important for amino acid for neurons. The proportion of glutamate converted to glutamine or oxidized in the TCA cycle seems to be dependent on glutamate concentration (226).

GABA released from presynaptic GABAergic neurons is taken up by astrocytes predominantly via SLC6A1 [GAT1]/ SLC6A11 [GAT3]. GABA is metabolized in the TCA cycle forming

glutamate and later glutamine (227). Glutamine is the only precursor molecule for the generation of amino acid neurotransmitters glutamate and GABA. As GLUL is predominantly expressed in astrocytes (228, 229) they serve as a critical source for glutamine synthesize. Glutamine is released from astrocytes via SLC38A3 [SNAT3]/ SLC38A5 [SNAT5] transporters and subsequently taken up and processed into glutamate and GABA in glutamatergic and GABAergic neurons, respectively (227).

Glutamate uptake assays are commonly performed for validating astrocytic functions of hPSC derived astrocytes (15, 16, 183, 230-234). However, model comparison and interpretation are difficult since experimental procedures vary substantially between publications in regards to glutamate levels in the culture media before the assay, washout time before assay initiation, normalization, control conditions, number of time points, assay read out, and transporter modulation. Multiple cell types can reduce extracellular glutamate levels such as HEK293 cells (15, 234). However, astrocytic glutamate uptake is often associated to SLC1A3 and SLC1A2 of the solute carrier 1 family [excitatory amino acid transporter, EAAT], constituted of five transporters SLC1A1-5. Since SLC1 transporters are sodium dependent several astroglia models can show that SLC1 transporters contribute to glutamate uptake by using Na^+ depleted control conditions (15, 230). Applying glutamate competitive inhibitors L-trans-pyrrolidine-2,4-dicarboxylic acid (PDC) (15, 230) or DL-threo-hydroxyaspartic acid (β-THA) (233) also demonstrates SLC1 dependent uptake in astroglia. However, further research is needed to stratify the relative activity of SLC1A2 and SLC1A3 to glutamate uptake in astrocytic models since they seemingly contribute differently *in vivo* (221). This can be performed using targeted pharmacological inhibitors (235). Moreover, additional characterization of the glutamate transport system including ion homeostasis via Na^+,K^+-ATPase, plausibly ATP1B2 and ATP1A2 in astrocytes, will further increase translability and relevance.

3.3.5.2 Reactive Response by Astrocytes

Astrocyte reactivity is a cellular response to neuropathologies including acute focal insults caused by traumatic injury and ischemic stroke as well as infections, neurodegenerative diseases, tumors, and autoimmune disease (207). Reactivity is a process to limit tissue damage, cell stress and restoring normal tissue homeostasis. The reactive response is not a homogenous single sided event, but is gradual and circumstantial, which can be linked to the type of neuropathology, characterized by brain region, niche, severity, and delimiting features (166).

Invasive focal insult activates astrocytes in its vicinity which start to proliferate forming a barrier around the injured site. The scar is often composed of high astrocyte density with elongated and intermingled morphology having cell bodies double the size of healthy astrocytes (236). The proliferative phenotypic response accompanied with a stem cell like potential is predominantly observed during invasive trauma and cerebral ischemia which is not observed in noninvasive injuries. Isolation of glia cells from the injury site form proliferative neurospheres in cultures having multipotency capacity. The proliferative phenotype of reactive astrocytes is partly linked to SHH signaling which can also induce similar effects on non-reactive astrocytes of the gray matter (237). Of note, isolation of primary astrocytes from non-

pathological conditions often select for proliferative population, which show multipotency if isolated from neurogenic niches in the adult brain (181).

There are a core set of transcripts upregulated in reactive astrocyte (166). However, large transcriptomic differences between neuroinflammation or ischemic stroke demonstrate injury specific astrocytic response, categorized as A1 and A2 astrocytes, respectively (166). Inflammatory response is also triggered by a variety of intercellular signaling molecules including proinflammatory factors such as TNF and IL1B originating from microglia, leukocytes and astrocytes (210). IL1A and IL1B stimulation show association of activating A1 and A2 reactive astrocyte, respectively. Additionally, activation of A1 reactive astrocytes is dependent on the presence of reactive microglia, releasing specific cytokines as TNF, IL1A and C1QA (208). One commonly used biomarker for reactivity is the upregulation of GFAP (191, 238), which has also been used on clinical samples where various GFAP isoforms where upregulated in Alzheimer's disease (AD) patients (191). Astrocytes also respond by upregulation of cytokine signaling genes (166) and secretion of cytokines (239) and other unknown factors shown to be neurotoxic (208).

Modeling astrocyte reactivity *in vitro* mimicking physical insult have been done using scratch tests (240). Modeling neuroinflammation response is performed by stimulation using various factors followed by measuring the secretion of cytokines indicating reactive activation (241). Pro-inflammatory response is used to validate model functionality of hPSC derived astrocytes. Increased IL6 and IL8 secretion in response to IL1B stimulation is observed for several hPSC derived astrocytic models (183, 242-245) as well as to TNF (242, 244, 245) and Ab42 (246) stimulation. This is in concordance with pro-inflammatory stimulation of human primary fetal astrocytes (244, 246). However, stimulation with lipopolysaccharides (LPS), trying to mimic bacterial infection, display contradictive results where stimulation of astrocyte models generates both non-inflammatory response (208, 245, 247) and an increase in IL6 secretion (246, 247).

Several hPSC derived astrocytic models studying reactivity (242-244, 246) and culture system for primary astrocytes (168) use FBS based conditions. A problem with investigating reactiveness of astrocytes *in vitro* is the fact that culturing conditions by it self can create a reactive stem cell like phenotype (20, 181) lowered by using defined condition (154, 208). This would imply that control conditions are not reflecting a non-reactive phenotypic state. The usefulness of a reactivity model would in such cases be dependent on the level at which the astrocyte is reactive in non-stimulated conditions. For example, if the astrocyte is at maximum reactivity before stimulation the inflammatory induction will not be representative, meaning that the stimulation response will depend on the basal level of reactivity during non-stimulated conditions. Several factors are associated with astrocyte reactivity including isolation procedure (248), extracellular matrix components (171), and the use of FBS (154, 155). New isolation and culture protocols maintaining a non-reactive state of primary human and mouse astrocytes demonstrate the importance of xeno-free and supportive cultures (154, 208, 248). Additionally, by applying defined conditions hPSC derived astrocytes differentiating via a glia

restricted progenitor (GRP) state demonstrate strong transcriptional changes after FBS exposure (20), which is also observed for primary GRP (155). However, withdrawal of FBS induced changes indicating a conversion back towards a non-reactive state (20). In accordance with FGF1 induced quiescence of FBS derived hPSC derived astrocytes (242) there are indication that a reactive state can at some level be reversible, but to what extent still needs to be determined.

3.3.5.3 Calcium Signaling in Astrocytes

Calcium signaling in astrocytes has gained increasing attention as it has shown higher importance to the neural circuits than historically believed (249). Astrocytes express a plethora of receptors (250) responsive to neurotransmitters such as glutamate, GABA, ATP and acetylcholine (ACh) which can induce an astrocytic calcium response. The endoplasmic reticulum (ER) is the major source for intracellular calcium which is an important second messenger for signal transduction. RYR3 and ITPR1/2 are the major calcium release channels expressed on the ER in astrocytes. Stimulation of G-protein couple receptors (GPCRs) can activate a downstream cascade where PLC cleave PIP2 forming IP3, which activate IP3 receptors in the ER releasing stored calcium into the cytosol (203, 251).

Astrocyte calcium oscillations related to synaptic transmission are not an all or nothing response, but dependent on synaptic transmission patterns which regulates oscillation amplitude in astrocytes (252). Neuronal transmission causing repetitive and sustained stimulation induce global calcium changes in the whole astrocyte (253). Calcium responses taking place in the soma are slow, seemingly contradictive for astrocytic regulation of fast and localized synaptic transmission. However, astrocytic response is more rapid in the fine processes (253). Astrocyte processes contain functional compartments enabling modulation of basal transmission, the release of neurotransmitters at individual synapses to a single action potential (252). Glutamatergic neuronal signaling, activating astrocytic GRM5 induced calcium signaling, can result in astrocytic release of adenosine which stimulate presynaptic ADORA2A receptors, modulating basal synaptic transmission (252).

Calcium signaling in astrocytes is associated with their functional maturation during development. Glutamatergic neuronal signaling via SLC17A7 [VGLUT1] induces GRM5 astrocytic calcium response (19). GRM5 expression is highest during development and decreased in adulthood (254). However, ablation of GRM5 induced calcium signaling reduces functional maturation and SLC1A2 expression. Additionally, a reduction of astrocytic domain development with arborization of astrocytic processes and lower synaptic enshealthing can be observed (19). Developmental maturation of calcium signaling response via GRM5 can also be observed for isolated human primary astrocytes. Fetal (GW17-23) and adult human astrocytes display calcium responsiveness to ATP stimulation but only adult astrocytes respond to glutamate stimulation. Pre-treatment with GRM5 antagonist, 2-methyl-6-phenylethynyl-pyridine (MPEP), abolishes glutamate calcium induction indicating a GRM5 driven calcium response in adult human astrocytes (154). Glutamate and ATP induce synchronous and asynchronous calcium response patterns, respectively. Moreover, addition of MPEP do not

affect ATP driven calcium signaling which together indicate different triggering mechanisms of calcium response (154).

ATP induced astrocytic calcium response can be activated via metabotropic P2Y1 and ionotropic P2X7 purinoceptors, and plausibly P2X2 and P2X4. However, ATP breakdown to ADP shows increased potency compared to ATP to trigger astrocyte calcium signaling whereas ADP breakdown to adenosine only triggers small astrocytic responses (255). Astrocytes can in turn propagate intercellular calcium signals by secretion of ATP and further activation of purinergic receptors on surrounding astrocytes (255). Calcium wave propagation is also observed via connexins, predominantly via GJB6 [Cx30] and GJA1 [Cx43] in astrocytes (187, 256). Interestingly, intracellular calcium waves working via gap junctions or purinergic receptors show compensatory mechanisms as ablation of GJA1 can induce a switch to function via purinergic receptors (257). Notably, studying physical dynamics of calcium wave propagation in relation to connexin transport and ATP release can be challenging in biological systems, but where mathematical modeling can provide interesting hypothesises (258). Astrocytic GJB6 can regulate excitatory synaptic transmission and the protrusion of astrocytic processes into the synaptic cleft (256). Intracellular astrocytic calcium signaling is linked to potassium homeostasis which is critical for neuronal activity and firing of action potentials (259). Astrocytes can sustain axonal activity by spatial buffering of potassium via potassium channel KCNJ10 [KIR4-1] (260). Moreover, by GCPR induced increase of cytosolic calcium, astrocytes can control Na^+,K^+,ATPase activity and dynamically regulate extracellular potassium levels resulting in decreased synaptic failure and increased synaptic fidelity (259).

Evaluation of calcium signaling is performed to functionally validate and assess the astrocyte biology of hPSC derived astrocytic models. Functional calcium signaling is shown in several hPSC derived models in response to mechanical (15, 16, 230, 242, 244), electrical (245), ATP (20, 183, 230) and glutamate simulation (246). Spontaneous calcium waves are also observed in hPSC derived models (183) together with calcium leakage from the ER, identified by inhibiting the major ER release channels RYR, IP3R and SERCA (245). Mechanically induced calcium wave propagation demonstrates ITPR mediated signaling dependent on ATP propagation (15) or gap-junction coupling (244). ITPR mediated signaling is also responsible for ATP stimulated calcium signaling in hiPSC derived astroglia (230). Notably, *in vitro* astroglia models often lack the complex morphology observed *in vivo.* This hinder to study the rapid calcium communication with neurons believed to occur in the fine branched processes as compared to slow calcium transients in the somata.

3.3.5.4 Investigation of Astrocyte Associated Functions using hESC Derived Astrocytes

Astrocyte functionality is diverse and several astrocyte-associated functions besides glutamate uptake, inflammatory response and calcium signaling are studied using hPSC derived models including; potassium conductance (15, 261), perineural net formation (262), oxygen consumption rates (245), phagocytosis (246), gap junctions connectivity (183) and metabolism (234). Notably, the development of astrocyte buffering functions of different ions is likely to be affected by medium composition where an imbalance might hinder this process. This can

be observed in neuronal cultures where classical basic medium and FBS impair electrical activity and synaptic communication (263). Moreover, hPSC derived astrocytes are being used to study astrocytic effects on neuronal biology including; synaptogenesis (183), neurite outgrowth (16, 20) and neuronal electrophysiology (183). Additionally, assessment of human isolated and PSC derived astroglia biology is studied by engraftment in rodents (8, 16, 29, 30, 183).

Establishing translational models of astrocyte biology provides the possibility to study astrocytes involvement in pathological conditions and diseases. Human isolated and PSC derived astroglia models are being used to study astrocytes involvement in AD (245, 264, 265), ALS (97, 230), Huntington's disease (HD) (266), Alexander disease (183, 267), Costello Syndrome (262) and Schizophrenia (268).

3.4 Astrocyte models

Establishment of *in vitro* models to study human astrocyte biology currently use primary tissue or hPSCs as source material. Isolation from primary tissue can either focus on the direct utilization to study fetal and mature astrocytes (154), glioma (269) and adult stem cells (270) or the isolation of progenitors for further astrocyte differentiation and maturation *in vitro* (155, 271). Human PSC derived astrocytic models apply differentiation protocols to direct hPSC towards an astrocytic fate after neuralization by using signaling factors (15, 20) and in combination with protein overexpression systems (16, 183).

3.4.1 Primary Glia

Primary astrocyte isolation methods have different methodology depending on the cell population to be targeted. Basic protocols focus on the proliferative capacity and attachment capabilities of isolated cells selecting for a mitotic population which expand over time and can easily be cultured (272). Besides genetically engineered reporter systems in mice, isolation of specific cell population from adult and fetal brain is predominantly based on protein expression or lack of protein using immunoselection (273, 274). Additionally, to isolate various cell types, a multistep immunopanning procedure can be applied where different cells populations are removed as part of the selection of the targeted cell population (154, 208, 248).

Supporting culture conditions are critical for cell survival and sustained phenotypes of primary cells. Growth factor FGF2 was used in early development of primary isolation methods of neural stem cells from mice which can drive NPC proliferation *in vitro* (53, 54). Combinations of FGF2 and EGF enable NPC isolation from both mouse and human primary tissue (58). Media using high FBS and glucose concentrations are used to increase survival and proliferation capacity after isolation. Purification of astrocytes from human fetal brain can be performed by continuous passaging to remove neurons and oligodendrocytes while free floating microglia are removed during medium changes (241). Isolation and selection between cell populations is also achieved by exploiting the cells potential to attach to different surfaces under static and shaking conditions (275). Focusing on proliferative properties enables the exclusion of FBS developing more defined conditions (155). More targeted isolation of human

glia restricted progenitors from fetal tissue is achieved by selection and counter-selection for a specific ganglioside ST8SIA1 detecting antibody A2B5 and for an epitope of a polysialic acid linked to NCAM1, respectively, which can also be recapitulated *in vitro* by hPSC differentiation (29, 30). Isolation from human adult tissue shows that tripotent stem cells reside in the adult brain (270, 276) which can also be utilized as a source for adult progenitor derived astrocytes (271).

To support and sustain mature *in vivo* like astrocytic phenotypes fully defined isolation and culture conditions have been developed. By screening several factors hbEGF and/or Wnt7a in contrast to generally considered neurotrophic and gliotrophic factors, such as GDNF, are necessary for mouse primary astrocyte survival (248). The same culture conditions are proven to work for human primary astrocyte isolation from fetal and adult tissue (154). Combining immunopanning with defined supportive media composition can isolate human astrocytes displaying high morphological similarities with more mature astrocytes *in vivo* (154). This protocol has also been applied for purifying brain organoid derived astrocytes (175). Isolating cells with an astrocytic phenotype is currently performed using immunopanning with a last step purifying the cell population based on expression of HEPCAM (154) and ITGB5 (208).

3.4.2 Human PSC Derived Astrocytes

To regulate NSC differentiation potency to become gliogenic and generate astrocytes include the governance of signaling pathways associated to astrocyte development. Astrocyte differentiation can be observed as different stages; inhibition of astrogenesis, acquisition of gliogenic responsiveness, astrocyte differentiation, and astrocyte maturation. Commonly associated pathways include FGF, JAK/STAT, BMP/SMAD, TGFβ, EGF, and Notch signaling (277, 278), which are applied in various combination to differentiate hPSC to astroglia (Table 1).

During recent years several publications have provided methods to differentiate human PSC into astroglia lineages. Many of these protocols depend on long-term cultures spanning up to 200 days (15, 264). Long-term protocols either use more defined conditions, culturing NPC in EGF and FGF2 (15), or in FBS (266), as well as with the combination of EGF, FGF and FBS (279). Expansion and generation of astroglia is done both in adherent (231, 266) and suspension cultures (15, 243). To improve differentiation purity and efficiency GFAP reporter cells lines have been established (243, 280) together with surface marker purification via FACS (Yuan, Martin 2013) and plastic attachment selection (264).

Roybon, *et al.* apply a long-term protocol for the generation of astroglia relying on extensive culturing of neuronal cells in FBS. The differentiation into astroglia display characteristic markers including CD44, GJA1, VIM, NF1A, ALDOC, SLC1A2 and with a high efficiency of GFAP (70%) and S100B (~100%) expressing cells. These cells also show functional properties in the form of glutamate uptake, calcium waves and reactive response phenotype to TNF and IL1B stimulation. Applying FGF1/2 at the end of the differentiation process can change the astroglia phenotype, reducing GFAP and NF1A expression, but keeping a

functional glutamate transport, suggested by the author to be a quiescent phenotype (242). The long-term FBS culturing methodology is also applied by others to differentiate patient specific hiPSC cells into astroglia with an ALS genotype (280, 281). Via long term cultures using FGF2 and EGF Krencik *et al.* generate astroglia displaying the characteristic marker profile S100B, GFAP, A2B5, CD44, NFIA. The astroglia demonstrate functional properties such as synaptogenesis promotion, glutamate uptake, and electrophysiological features including calcium wave propagation and decrease voltage dependent outward currents when co-cultured with neurons. At the end of the differentiation, after 180 days, addition of LIF or CNTF increase the percentage of GFAP/S100B positive cells, staining approximately 90% of the population (15). Defined conditions with an EGF and LIF intermediate step before CNTF maturation can shorten the protocol down to 80 days (20). Applying the maturation factor combination of BMP2/4 and LIF reduced time lines slightly further down to 70 days (231). Progenitor expansion in neurosphere format before astrocyte maturation also report shorter time lines (230, 282). Differentiation of NPC using FGF2 and EGF long term culture show cellular developmental stages associated to early (d14 E-RG) and midneurogenic radial glia (d35 M-RG) followed by outer and gliogenic radial glia (d80 L-RG) which at day 220 associate to adult-like progenitor cells (283). Late RG display loss of epithelial integrity and increased association to SVZ and oRG progenitors. Transcripts *OLIG1* and *PDGFRA* at day 80 associate to HES5+ cells, which substantially decrease during late development while SLC1A3, FABP7, EGFR, and S100B increase in both HES5+/- populations. Interestingly, differentiation potency to GFAP+ cells upon FBS stimulation was only observed from HES5+ L-RG. It demonstrates that astroglia expression profile of FABP7, SLC1A3 and S100B can develop via a GFAP negative progenitor state. This study provides insightful biological translation for astroglia differentiation protocols using long-term FGF2 and EGF expansion. Notably, astroglia protocols often include repetitive passaging which will purify mitotic populations where frequency of passaging will further select populations based on their proliferation rate.

Shortening the laborsome long-term culturing protocols for the generation of astroglia would be most beneficial and is currently being developed by several groups. FGF2, used in the long-term differentiation methods, is a common factor also in the majority of shorter astroglia differentiation protocols. However, the application of other signaling factors reduce the differentiation time lines down to 20-40 days from an initial NPC stage (233, 261). Notably, the definition of an astrocytic phenotype is variable between publications, making time lines context dependent. Majumder *et al.* modulate the epigenetic landscape by using chromatin modifiers including Aza-C and TSA together with BMP2 to achieve faster differentiation. Methylation arrays shows that astroglia related markers GFAP, S100B, AQP4 are hypermethylated in NSC. Combining Aza-C and TSA with BMP2 more efficiently differentiate NPCs into GFAP (20%) and S100B (80%) expressing cells within 15 days, which demonstrate decreased methylation pattern on astrocytic gene promoters. Additionally, markers such as ALDH1L1 and SLC1A3 could be detected, but no functional evaluation was performed on this population (284). Direct application of astrocytic factors CNTF, JAG1-Fc, and CT1 on isolated NPC rosette structure significantly increase the GFAP+ population within

35 days but still compose of a high number of βIII tubulin positive neurons (285). Shaltouki *et al.* can corroborate the generation of astroglia by CNTF and BMP2 stimulation, but the condition including a neuregulin splice variant, heregulin, show the highest differentiation efficacy. Differentiating NPC for 35 days, via a CD44 positive stage, results in 69-80% GFAP positive cells. These cells exhibit glutamate uptake functionality together with synaptogenesis promotion when directly co-cultured with hESC derived neurons. (233). After 28 days FGF2 and BMP4 induction Lin *et al.* demonstrate by using immunoselection for SLC1A3 that a highly enriched S100B+ population can be achieved after 14 days of FBS exposure (286). Fully defined xeno-free system using FGF2 and BMP4 media on PLO-Fibronectin coating can also generate astroglia within 20-40 days (261)

Goldman's group has generated a protocol for hiPSC derived OPCs and oligodendrocytes, which can be applied to generate astroglia even though the differentiation process develops via an oligodendrocyte associated marker expression profile, including OLIG2, NKX2.2, SOX10, A2B5, PDGFRA. The protocol is around 120-200 days, which can generate astrocytes and oligodendrocytes, both *in vitro* and *in vivo* upon transplantation in mice (8). Similar approach differentiating via a glia restricted progenitor state expressing A2B5 is performed by several hPSC derived astrocytic protocols (244, 261).

Screening the differentiation efficacy of several of the previously mentioned astrocyte differentiation mediums was performed by TCW *et al.* across 42 NPC lines (246). Quality was assessed by GFAP, S100B, morphology, survival, proliferation, reproducibility and cell lines variation. A non-defined commercial media in combination with 2% FBS met the desired criteria. The protocol generated highly proliferative astrocytes with astrocytic morphology expressing GFAP, S100B, SLC1A3, ALDH1L1, and APOE (246). The authors emphasized that the protocol efficacy depended on the quality of the NPCs (246), preferably having a PROM1 [CD133]+/ CXCR4 [CD184]+/ NGFR [CD271]- expression profile as previously described (287), in addition to strict single cell culturing during the first 30 days of the differentiation (246).

Shortening and simplifying the generation of astrocyte models is also achieved by genetic techniques overexpressing SOX9, NFIA and NFIB in hPSC generating iAstrocytes (16, 183). Overexpression is combined with standard expansion conditions of FGF2 (16, 183), EGF (16) and FBS (183) followed by maturation factors including BMP4, CNTF (16, 183), dbcAMP and hbEGF (183). Using stable integration in the AAVS1 locus of doxycycline inducible construct (16) compared to lenti-viral infection (183) iAstrocytes can be regionalized before astrocyte specification by differentiating hPSC to NSC using standard dual SMAD inhibition under patterning condition (16). Using genomic techniques to drive glial differentiation shortened the hPSC derived astrocyte protocol from 6 months (15) to 52 days (16). However, using lenti-viral expression with antibiotic selection generate homogenous iAstrocytes culture within 28 days from an hPSC state (183).

Generating regional specific astrocyte can be performed in similar fashion as for hiPSC derived neurons by patterning NSC before neural differentiation. Anterior-posterior patterning

generating OTX2-HOXB4 NPCs, respectively, persisted upon astroglia differentiation. Differences in calcium wave propagation distances can be observed between anterior-posterior astrocytes which otherwise show similar phenotypes (15). Lack of phenotypic differences between midbrain and spinal cord astrocytes is also observed in other protocols (243), which in general show small differences in GFAP and S100B expression. A recent study shows that the exposure or non-exposure to FBS is more discriminating than cellular origin (20), indicating plausible difficulties studying regional variances using FBS based differentiation protocols. This is indicated in the generation of Olig2-Astro which show large similarities with non-directed patterning NPC-Astro. However, an intermediate A2B5+ stage define a developmental difference between the two models together with functional oxidative protection capabilities. Exposure to FBS instead of BMP4 and FGF2 for 20 days ablate the A2B5 intermediate stage (261). The use of FBS during derivation of astrocytes is commonly applied, but recently there is a debate concerning the effects of FBS and *in vivo* representation. Application of FBS to non-exposed cells have shown phenotypical changes (20, 154, 155, 288). However, FBS induced effects has also been shown to be reversible (242), but to want extent and if FBS causes permanent changes is still unclear.

In vitro culturing conditions for astrocytes needs to be strictly defined to create reproducible and biological relevant astrocytic models since astrocyte biology has been shown, besides differentiation factors, to be affected by FBS addition (154, 155, 288), glucose levels (289) glutamate levels (290), basal medium composition (263), matrix proteins (171), and oxygen tension (291, 292).

3.4.3 Human PSC Derived Organoids in the Generation of Astrocytes

Recent technical development provides the possibility to generate brain organoids from PSCs which recapitulate brain structure, regionalization and cell diversity observed *in vivo*. This complex modeling system enables researchers to study unique features of human brain development, not fully captured in mice and non-human primate models (293). Organoids capture developing astrocyte around day 60 in culture (294), which over a longer time course of 180 days increase in abundancy (288). Isolated under defined serum-free conditions organoid derived GFAP+ astrocytes show stellate mature morphology (288). Stellate morphological cells transform into a polygonal shaped cells upon FBS stimulation while increasing markers of astrogliosis, demonstrating reactive responsiveness at this time point (288). Extending culture time of organoids to 590 days *in vitro* shows development and maturation of astrocytes resembling human primary mature adult astrocytes (154, 175). Immunopanning isolation of HEPACAM+ astrocytes from organoids spanning from 100 days to 495 days show increasing transcriptional identity with adult astrocytes in parallel to decreasing association with fetal stages. Mature organoid derived astrocytes show reduced proliferation and increased morphological complexity accompanied with acquisition of astrocyte associates functionality including SLC1 dependent glutamate uptake, phagocytose synaptosomes, induction of synaptogenesis, and effect on neuronal calcium signaling (175). Single cell analysis of 590-day organoid glial cells reveals population of cells at different

developmental stages. Transcriptional transition can identify vRG, oRG, fetal and adult astrocyte over the culture period. These share a considerable overlap making it interesting from a lineage tracing perspective to try and identify the origin and developmental pathway of human astrocytes (148, 154, 175). Common approaches to generate organoids start with culturing of PSC or NSC. However, with the focus to study the dynamics of astrocyte networks and neurons Krenick *et al*. developed organoid spheres of pre-differentiated hPSC derived astrocytes called asteroids. Combining asteroids and iNeurons triggers the development of a more complex astrocyte morphology and increasing synaptic density. This model might enable the study of the tripartite synapse with higher translability (34).

Table 1 - Differentiation protocols of hPSC derived astrocytes

Reference	Culture Type	Differentiation Approach	Key Factors	Matrix	Efficacy marker (%)	Time from NSC	Markers	Validation Methods
Emdad *et al.* 2012 (285)	Mono-layer	Pre-exposure during rosette formation and immediate differentiation on isolated neural rosettes in monolayer format	EGF, FGF2, CNTF	Matrigel, PLO/ Laminin	GFAP+ (78%)	35d	Nestin+, A2B5+	Immunostaining, migratory capacity
Majumder *et al.* 2013 (284)	Mono-layer	Monolayer differentiation modulating the epigenetic status using DNA methyltransferase inhibitors	BMP2, Aza-C, TSA	N.S.	S100B+ (80%), GFAP+ (20%)	30d	S100B+, CD44+, SLC1A3+, ALDH1L1+	Immunostaining
Shaltouki *et al.* 2013 (233)	Mono-layer	Monolayer culturing and differentiation	FGF2, BMP, CNTF, (1%FBS)	PLO/Lam or geltrex	GFAP+ (34%)	35d	S100B+, CD44+	Immunostaining, transcriptome profiling, synaptogenesis promotion, glutamate uptake
	Mono-layer	Monolayer culturing and differentiation	FGF2, ActivinA, Heregulin, IGF1	PLO/Lam or geltrex	GFAP+ (62%)	35d	S100B+, CD44+	Immunostaining, transcriptome profiling, synaptogenesis promotion, glutamate uptake
Jiang *et al.* 2013 (261)	Mono-layer	Directed monolayer differentiation	FGF2, BMP4	PLO/ Fibronectin	GFAP+ (94%), S100B+ (95%)	20d	A2B5+, VIM+, CD44+	Immunostaining, synaptogenesis promotion, glutamate uptake, electrophysiology, oxidative protection
	Mono-layer	Directed monolayer differentiation	FGF2, BMP4	PLO/ Fibronectin	GFAP+ (96%), S100B+ (95%)	20d	VIM+, CD44+	
Chen *et al.* 2014 (232)	Mono-layer	Directed monolayer differentiation	FGF2, BMP4	PLO/ Fibronectin	GFAP+/ S100B+ (95%)	20d	A2B5+, CD44+, VIM+	Immunostaining, glutamate uptake, electrophysiological neuron effects (Cond.Med.)

Krencik *et al.* 2011 (15)	Astro-spheres / Mono-layer	Conversion of neurosphere to astrospheres by extensive culturing with reduced sphere size followed by monolayer maturation	EGF, FGF2, CNTF	PLO/ Laminin	S100B+/GFAP+ (90%)	90-180d	A2B5+, CD44+, NFIA+,	Immunostaining, synaptogenesis promotion, glutamate uptake, calcium waves, electrophysiology
Gupta *et al.* 2012 (231)	Mono-layer	Extensive NSC culturing (55d) proir to astrocyte differentiation	EGF, FGF2, BMP4, LIF	Matrigel	GFAP+ (95%), S100B (90%)	67d	AQP4+ (79%), SLC1A3 -(89%)	Immunostaining, glutamate uptake
Sareen *et al.* 2014 (282)	Neuro-spheres	Gliogenic EZ spheres were caudalized and subsequently expanded in suspension culture before alternative final differentiation in adherent cultures	EGF, FGF, RA	N/A	GFAP+ (55%), S100B+ (75%)	21d	A2B5+, NESTIN+, ALDH1L1+, S100B+	Immunostaining
	Mono-layer	Gliogenic EZ spheres were dissociated and plated during caudalization and subsequently expanded in adherent culture before alternative final differentiation	EGF, FGF, RA	Matrigel, PLO/ Matrigel	GFAP+ (~20%), S100B+ (~5%)	80d	A2B5+, NESTIN+, ALDH1L1+	Immunostaining
Lafaille *et al.* 2012 (279)	Mono-layer	Extensive NSC culturing (60d) proir to astrocyte differentiation	EGF, FGF2, FBS	PLO/ Laminin	GFAP+ (90%)	70d	-	Immunostaining
Juopperi *et al.* 2012 (266)	Mono-layer	Extensive cultures in FBS	FBS	PLO/ Laminin	GFAP+ (~100%)	90d	S100B+	Immunostaining
Roybon *et al.* 2013 (242)	Mono-layer	Directed monolayer differentiation with patterning cues followed by extensive cultures in FBS, and an alternative maturation phase	FBS	PLO/ Laminin	GFAP+ (60-70%), S100B+ (~100%)	97d	CD44+, GJA1+, VIM+, NF1A+, ALDOC+, SLC1A2+	Immunostaining, glutamate uptake, calcium waves, factor secretion

Holmqvist *et al.* 2015 (243)	Neuro-spheres/ Mono-layer	Extensive cultures of neurospheres and monolayer differentiation with possibilities for FACS puification of reporter construct (GFAABC1D::RFP)	FBS	PLO/ Laminin	GFAP+ (~100%)	130d	CD44+, GJA1+, NF1A+, S100B+, GS+	Immunostaining, Inflammatory Stimulation, a-synuclein processing
Li *et al.* 2015 (281)	Mono-layer	Extensive cultures in FBS	FBS	PLO/ Laminin	GFAP+ (60%)	>90d	VIM+, S100B+, CD44+, SLC1A2+, SLC1A3+, AQP4+	Immunostaining
Zhang *et al.* 2015 (280)	Mono-layer	Extensive cultures in FBS followed by FACS purification of reporter construct (GFAP::GFP, AAVS1)	FBS	N.S.	GFAP+ (28-48% from FACS)	>90d	ALDH1L1+, S100B+, NF1A+, SLC1A2+	Immunostaining
Yuan *et al.* 2011 (296)	Mono-layer	Spontaneous differentiation of FACS purified NPC in monolayer cultured followed by glia purification via FACS	FBS	PLO/ Laminin	GFAP (N.S.), Nestin (High Level)	42d	FACS (CXCR4+/CD44+)	Immunostaining
Kondo *et al.* 2013 (264)	Mono-layer	Neuron differentiation of NPC followed by extensive culturing and selection by passaging on non-coated culture plates	FBS	Matrigel, Non-coated, Gelatin	GFAP+ (80-95%)	>200d	ALDH1L1, VIM, SLC1A3, APOE	Immunostaining, reactivity response, phagocytic capacity, calcium signaling
TCW *et al.* 2017 (246)	Mono-layer	NSC (CD271-/CD133+/184+). Seeded spars at 15K/cm2 and	ScienCell (AM) + FBS	Matrigel	GFAP+ (82%), S100B (90%)	30d	ALDH1L1, VIM, SLC1A3, APOE	Immunostaining, reactivity response, phagocytic capacity, calcium signaling

Santos *et al.* 2017 (244)	Mono-layer	Differentiation directly towards A2B5+ glia progenitor without NSC stage following FBS+LIF maturation on non-coated plates	PDGFAA, FGF2, EGF, FBS, LIF	PLO/ Laminin + Non-coated	GFAP+ (>95%), S100B (>95%)	49d	A2B5, NFIA, ALDH1L1, CD44, SLC1A2, SLC1A3	Immunostaining, calcium signaling, glutamate uptake, gap junction propagation, Inflammatory Stimulation, neuron/astrocyte co-culture
Oksanen *et al.* 2017 (245)	Neuro-spheres / Mono-layer	Long-term expansion of NSC before astrocyte maturation	Heparin, FGF2, EGF, CNTF, BMP4	Matrigel	GFAP+ (90%), S100B (90%)	>200d	--	Glucose uptake, Glutathione secretion, Calcium Signaling, Oxygen consumption rate, Oxidative stress, Neuron-Astrocyte co-culture
Perriot *et al.* 2018 (20)	Mono-layer	Differentiation via glia progenitor and astrocyte glia progenitor before astrocyte maturation.	FGF2, EGF, LIF, CNTF	PLO/ Laminin, Matrigel	GFAP (50%), S100B (>94%)	72d	SLC1A3	Inflammatory Stimulation, glutamate uptake, neurite outgrowth, calcium signaling
Serio *et al.* 2013 (230)	Neuro-spheres / Mono-layer	Conversion of neurosphere to astrospheres by culturing with reduced sphere size followed by monolayer maturation	EGF, LIF, FGF2, CNTF	Matrigel	GFAP+ (90%)	>56d	VIM+, NFIA+, S100B+	Immunostaining, synaptogenesis promotion, glutamate uptake, calcium waves

A2B5 (also known as ST8SIA1), ALDH1L1 (Aldehyde dehydrogenase 1 family member L1), AQP4 (Aquaporin 4), Aza-C (5-azacytidine), BMP2 (Bone morphogenetic protein 2), BMP4 (Bone morphogenetic protein 4), CD133 (also known as PROM1), CD184 (also known as CXCR4), CD271 (also known as NGFR), CNTF (Ciliary neurotrophic factor), CX43 (Connexin 43, also known as GJA1), EGF (Epidermal growth factors), FBS (Fetal bovine serum), FGF2 (Fibroblast growth factor 2), GFAP (Glial fibrillary acidic protein), GFAP (Glial fibrillary acidic protein), GS (Glutamine synthetase, also known as GLUL), LIF (Leukemia inhibitory factor), NF1A (Nuclear factor I A), O4 (Forkhead Box O4), PDGDAA (Plate derived growth factor AA), PLO (poly-L-ornithine), RA (Retinoic acid), S100B (S100 calcium-binding protein B), SLC1A2 (Solute Carrier Family 1 Member 2, also known as EAAT2, GLT-1), SLC1A3 (Solute Carrier Family 1 Member 3, also known as EAAT1, GLAST), NSC (neural stem cells), TSA (Trichostatin A), qRT-PCR (quantitative real-time polymerase chain reaction), N.S. (Not Specified), N/A (Not applicable)

4 Human iPSC derived Neural *In vitro* Models in Pharmaceutical Development

Since the discovery of the iPSC technology the number of human iPSC derived neural models used in pharmaceutical screening settings has increased dramatically. Not to be neglected, circumventing the ethical aspects of using embryonic stem cells by applying iPSC has enable new possibilities. As the differentiation protocols improve the generation of more specific and functional models the application and investigation of specific biological traits using stem cell derived models increase. Screening settings can thereby look at disease mechanism linked to specific cellular subtypes such as dopaminergic neurons in PD. Another key factor in the utilization of stem cell derived neural models is the accessibility of the cell. Large compound screens require large number of cells. This has previously been circumvented using immortalized cell lines or applying primary cells later in drug development when validating desired properties of a reduce number of candidate compounds. By using hiPSC derived neural models the number of cells is not a problem. Additionally, genetic modifications are more easily performed in PSCs compared to primary cells which often have a limited number of division and are harder to transfect. This enables the generation and correction of genetic disease backgrounds in addition to the availability of genetic cohorts using hiPSC. The use of stem cell models could potentially improve drug target identification and optimize stratification of candidate compounds in the hope of achieving higher translability making clinical trials more efficient. Even though PSCs derived models present new challenges such as fetal like phenotypes, differentiational variability between lines and that there are not protocols for all cell types, yet they have shown their usefulness as models, particularly in neural systems where the supply of primary human cells is very limited. However, there is always a concern if the mechanism of disease and therapeutic candidate have any *in vivo* relevance, a translational gap that might be reduced with the utilization of stem cell derived complex physiological systems.

The drug discovery process is commonly initiated by applying a diversified or selected compound library in a primary screen utilizing a model with a desirable read-out. Hit identification is followed by lead series generation which optimize compound target efficacy. Additionally, chemical optimization tries to remove undesired activity identified in a secondary/counter screen, for example minimizing off target effects. This is critically important when progressing with lead series compound minimizing late stage failures in more costly experimental set ups, or clinical development.

4.1 Human iPSC Derived Neuronal Models in Screening

Human iPSC derived neurons have been used to study disease (297) such as AD (21), PD (73, 298), and HD (299). Additionally, as derivation protocols become more robust providing culture homogeneity and large-scale production hiPSC derived neurons are being utilized in compound screening of increasing size (22, 23). Safety issue is one of the major causes of drug projects closure, both in preclinical and clinical stages (300) signifying the need to selectively remove compounds displaying unwanted effects early in drug discovery programs. Human iPSC derived neurons are being applied in neurotoxicity screening (23) and used to study

compounds affecting neurite out growth (22) contributing to safety selectivity in compound development progression. Applying hiPSC derived neurons for studying disease, function and safety could play a part in improving the decreasing number of CNS programs in drug development (301).

4.1.1 Removing Unwanted Effect of Plasminogen Binding Inhibitors

Upon damage to blood vessels, platelets form a plug at the site of injury in parallel to depositing mature fibrin by the conversion fibrinogen minimizing blood loss in the formation of a fibrin clot. Restoration of tissue function involves fibrinolysis, degradation of the fibrin clot. This is performed by plasma circulating plasminogen which anchor to fibrin by kringle 1 binding to plasminogen. Plasminogen unfolds once bound to fibrin and is converted into active plasmin by tissue-plasminogen activator. Plasmin then cleaves fibrin generating fibrin degradation products and subsequent removal of the fibrin clot (302).

However, in the clinic, major surgeries requiring blood transfusion are associated to hyperfibrinolysis initiating unwanted bleeding which can causing severe effects. Thereby, antifibrinolytic drugs are used to reduce mortality following major trauma as well as reduce the need for blood transfusion and minimizing the risk of reoperation due to bleeding (303-305). Fibrinolysis can be reduced by plasminogen binding inhibitors (PBIs) which act to prevent kringle binding to plasminogen inhibiting its anchoring to fibrin and subsequent maturation to plasmin (306). PBIs are currently used and administrated in the clinic (303-305). However, commonly used plasminogen binding inhibitor TXA is associated with an increased risk of seizures (305, 307). This has been associated to an unwanted selectivity towards inhibitory neuronal receptors $GABA_A$ and glycine (307-309) which is also observed for more potent inhibitors as 4-PIOL (310). To identify PBIs which do not show $GABA_A$ receptor activity potent inhibitors are counter screened for this effect (311).

Receptor functionality in neurons is of major interest in the pharmaceutical industry. Studying receptor functionality in hiPSC derived neurons is performed by using various techniques such as patch clamping (312), calcium imaging (313), MEA, used to study neuronal circuit-connectivity (314). One of the previous techniques in the pharmaceutical industry to measure binding activity to neuronal derived receptors is based on isolation of neuronal rat membrane (310, 311). This requires animal and is both time consuming and does not show compound mode of action, if it behaves as an agonist or antagonist. Human iPSC derived neurons having a transcriptomic profile associated to fetal prefrontal cortex showed functional ionotropic glutamate receptors observed by dose dependent response to AMPA and NMDA administrations (313). In addition, the hiPSC derived neuronal model shows immature $GABA_A$ receptor functionality by calcium response to GABA and gabazine administration (313). Patch clamping techniques show similar $GABA_A$ receptor functionality in response to GABA and inhibition by bicuculine (312). Patch-clamping also demonstrate functional glycine receptors in hiPSC neuronal cells partially inhibited by picrotoxin indicating heteromeric receptor composition (315). A common problem with previous techniques is the low-throughput relative to compound screening. To increase throughput in functional studies automated patch-

clamping can be used, this technique shows a 40% success rate of measured cells, providing comparable concentration response curves (312, 316). Optical biosensors that detects refractive index alterations due to dynamic mass redistribution is another potential high-throughput technique to study receptor functionality (317, 318), which together with an additional label free technology is commonly used to study GPCRs (319). By activating the $GABA_A$ receptor of a neuroblastoma cell model, live cellular responses are measured by translocation of cellular mass demonstrating corroborating concentration response curves to GABA stimulation (320).

Besides studying development and disease mechanistic pathways hiPSC derived neural models have a great potential and applicability in pharmaceutical development to identify and avoid unwanted functional effects of lead compounds. This can lead to more efficient lead series optimization and effective generation of candidate drugs with the goal of minimizing the risk of patient health.

4.2 Human Astrocytic Models in Screening

Astrocytes are implicated to contribute to various diseases and pathophysiological conditions (321) making them a reasonable target for therapeutic intervention. Drug induced effects have been studied in various human astrocytic models including CCF-SSTG1 (269, 322), primary fetal (269, 322, 323) and human PSC derived astrocytes (23, 264, 324). Screening using glial models has commonly focused on treatment against glial tumors, either by using cells expanded from the primary tumor (325), reprogrammed cancer cells (326) or by genetic modifications of hiPSC derived glia inducing cancerous phenotypes (327). Combining glioblastoma like cells with normal stem cells, a counter-selection of compounds can be made identifying hits affecting glioblastoma like cells but not healthy stem cells (327, 328). Moreover, for drug induced effects of astrocytic models, several studies have focused on phenotypic readouts of reduced oxidative stress (323), neurotoxicity (23), APOE secretion, (269, 322), and AD (264). A larger number of evaluated compounds is observed in a few studies using hiPSC derived or human primary astrocytes (23, 269, 322, 324).

4.2.1 APOE Biology in Relation to Alzheimer's Disease

Lipid and cholesterol transfer between cells is facilitated by APOE serving as a ligand to endocytic mediated uptake via LRP1 and LDL receptor family expressed on neurons, microglia and astrocytes (329, 330). APOE is predominately expressed by astrocytes (331) therefore having an important role in regulating cholesterol homeostasis. There are three APOE isotypes (APOE2/3/4) of which APOE4 is a strong genetic risk factor for developing sporadic AD (332) contributing to the majority of AD cases compared to familial AD which is linked to specific mutation in the APP and PSEN1/2 genes (333). Molecular associations of AD are increased load of Amyloid-β (Aβ) peptide composition and aggregation in combination with Tau accumulation (334). APOE3 compared to APOE4 code for cysteine and arginine at the amino acid position 112, respectively. Arginine affects protein structure affecting APOE4 function, reducing binding of cholesterol and lipids (335) and susceptibility to proteolytic cleavage (336). The different APOE isotypes are associated to increased Aβ accumulation in increasing

order APOE2<APOE3<APOE4 (337) while lipidated APOE-Aβ binding correlates inversely (338) indicative of APOE association to Aβ clearance. APOE affects Aβ clearance by cellular uptake (339) degradation (340) and receptor-mediated transport across the BBB (341). Decreased deposition is linked to increased APOE lipidation regulated by ABCA1 function which mediate cholesterol efflux (342). APOE4 and ABCA1 loss of function mutation have a hazard ration for AD of 7.70 and 4.13, respectively, indicating its relevance in AD development (332). On this line AD mouse models show that LXR and RXR agonist stimulation regulate APOE and ABCA1 expression levels influencing AD pathology (343). Genetically modified AD rodent models provide insight in AD biology and regulation but since large number of drugs have failed to reach the clinic (344) discussions on how to interpret the outcomes from these rodent AD models (345) and the role of APOE in disease and as therapeutic target (333) are ongoing.

4.2.2 Screening for Increased Secretion of Astrocytic APOE

Biological features associated to AD are observed in hiPSC derived astrocytes (264, 265, 346) displaying both oligomeric Aβ accumulation in relation to AD genotypes (264) and functional ability to secrete Aβ and soluble APPα (346). Additionally, hiPSC derived astrocytes generated from an APOE4 genotype display phenotypic association with PSEN1 genotype compared to healthy control (265). Moreover, APOE4 and an isogenic control APOE3 display distinct differential APOE protein expression, cholesterol binding and secretion, Aβ uptake and lysosomal degradation in hiPSC derived astrocytes together with increased Aβ association to synaptic puncta in organoid models (286).

Mice AD models show reduced Aβ levels and improved memory in association with increased APOE expression during agonist treatment of LXR and RXR (343). Additionally, Aβ degradation is dependent of APOE lipidation (340) which can be regulated by ABCA1, increased by LXR/RXR agonists. The strategy to increase APOE and/or lipidation levels might be a therapeutic possibility. Screening for APOE enhancing agents using human astrocytoma cell line CCF-STTG1 and human fetal astrocytes confirm upregulated expression of APOE during treatment with LXR agonists GW3965 and T0901317 (Tularik) (269, 322). However, as these agonists cause unwanted effects such as hypertriglyceridemia (347) Fan *et al.* could identify a LXR transactivating compound with lower SREBP-1c induction but still increased ABCA1 and APOE expression, thereby likely reducing hepatotoxic effects observed with traditional LXR/RXR agonists (322). Further development identified APOE and ABCA1 enhancing P2X7 annotated compounds with low SREBP-1c induction through an indirect LXR mechanism (348). Using a reverse approach Finan *et al.* applied human fetal astrocytes in the primary screen which demonstrated a mechanism of increased APOE secretion without ABCA1 induction (269). These compounds affect enzymes in the cholesterol biosynthesis pathway DHCR7 and DHCR24 (269) which show decreased levels in neurons of affected AD brain regions (349). DHCR24 mediates lipid raft formation and is a target gene of LXRα (350). Interestingly, these compounds increased APOE levels in primary astrocytes but not in the astrocytoma cell lines (269). This demonstrates that cellular context affects hit finding,

highlighting the risk and complexity of excluding compounds based on the primary screen. If the astrocytoma cell line would have been used in the primary screen of Finan *et al.*, DHCR7 and DHCR24 would not have been identified and confirmed in a secondary screen using primary astrocytes since they would have been removed in the first selection.

However, despite the efforts of finding compounds increasing APOE secretion there is a debate weather targeted treatment of APOE4 genotype should lower or increase APOE expression since it is not clear if APOE4 results in loss of function or gain of toxic function. Additionally, transcriptional changes between APOE3 and APOE4 relating to metabolic processes (286) might indicate that compound potency or toxicity between genotypes is not the same such that hit findings should be performed or validated in APOE4 genotypes. A contribution to this discussion is a study demonstrating that by using human recombinant APOE2/3/4 stimulation of hiPSC derived neurons, APOE can function as a signaling molecule to increase APP expression (351). Moreover, APOE4 demonstrated higher potency for signaling activation compared to APOE3 and APOE2 in their delipidated forms (351).

5 Aims of Thesis

The overall aim was to apply hiPSC derived astrocyte and neuronal models in a pharmaceutical screening setting.

The specific aims were:

- to develop a fully defined protocol for ltNES derived astrocytes demonstrating astrocytic translability on a transcriptomic, proteomic and functional level (adressed in paper I)
- to characterize current astrocytic models available for the pharmaceutical industry (adressed in paper I)
- to apply the astrocytic models in a pharmaceutical assay setting (adressed in paper I)
- to apply hiPSC derived neruons in a pharmaceutical assay setting (adressed in paper II)
- to develop a completely defined protocol and remove all animal components to increase robustness (adressed in paper I)
- to study the development of astrocyte biology *in vitro* to increase the understading and level of *in vivo* translability (adressed in manuscript III)

6 Results Summary and Discussion

6.1 Paper I:

The establishment of a long-term neuroepithelial-like stem (ltNES) cell (61, 62) having a homogenous and stable phenotype over extended culture time provide a valuable cell source for subsequent studies. By using ltNES cells as starting material, experimental timelines are shorted by several weeks in addition to higher reproducibility and robustness. Translating to neuroepithelial stem cells before GW7 the neurogenic ltNES model has predominantly been used to characterize spontaneous neuronal differentiation (56, 62, 352). In Paper I we show that ltNES can acquire a gliogenic potency by the protein expression of NFIA and SOX9, key transcription factors for glia onset. Furthermore, expression of BLBP, S100B, SLC1A3 and SLC1A2 together with several transcriptomic markers *ALDH1L1*, *AQP4*, *TNC*, *RYR3 ATP1B2*, *ATP1A2*, *GRM5* strongly indicate that ltNES cells attain an astroglia fate (NES-Astro). We then wanted to benchmark the NES-Astro model against other astrocytic models in the pharmaceutical industry including astrocytes from various sources; primary adult tissue (phaAstro), astrocytoma (CCF) and another commercially available hiPSC derived astrocyte (iCellAstro). Notably, an important aspect is that the pharmaceutical industry due to ethical considerations, is very restricted in the use of fetal derived models. This means that the most logical comparison between NES-Astro and fetal astrocytes, commonly used in the research field (20, 269, 322), is not included among the evaluated models. However, to identify neural and astrocyte associated characteristics we included ltNES (neural representation) and HEK293 cells (non-neural representation). Based on transcriptomic and protein expression we could detect large differences between the six models. However, since there is no reliable marker or transcriptomic identity fully specifying astrocyte biology we wanted to assess several functional properties associated to astrocytes *in vivo*.

Removal of excess glutamate in the synaptic cleft is a critical astrocytic function to avoid excitotoxicity and keep synaptic homeostasis during neuronal transmission. This is achieved by glutamate transport from sodium dependent transporters SLC1A3 and SLC1A2. In this test we can demonstrate NES-Astro having an active SLC1A3 dependent glutamate uptake over time, not observed for any of the other models. Moreover, astrocytes can display an inflammatory response in the event of brain trauma. We simulated this by treatment with pro-inflammatory cytokines TNF and IL1B evaluating the response by secretion of IL6 and IL8 as previously studied (244, 246). We could detect a dose dependent response in the NES-Astro model significant from ltNES. Inflammatory response was also observed for the other models. Interestingly, the baseline of IL6 and IL8 was high in phaAstro, CCF and iCellAstro while no secretion was detected from NES-Astro, indicative of a completely inflammatory inactive state. This can plausibly be linked to xeno-free and FBS based culturing condition for NES-Astro and the other astrocytic models, respectively, since FBS is known to induce astrocytic inflammatory pheontypes (20, 154, 166). Calcium signaling in response to neurotransmitter release is another important function of astrocytes for downstream regulation and feedback to neurons and neighboring astrocytes. Using a fluorescent $Ca2^{+}$-dye we could monitor calcium

fluctuations in response to ATP and glutamate. NES-Astro and phaAstro could show ATP response while only NES-Astro responded to glutamate stimulation. Interestingly, in one biological replica, C1, there was no significant difference in the number of ATP and glutamate responding cells enabling us to investigate the calcium response patterns. This could show asynchronous and synchronous signaling in response to ATP and glutamate, respectively, corroborating the findings observed from fetal and adult astrocytes using a refined isolation method preserving *in vivo* phenotypes (154, 208). Additionally, an initial observation was that NES-Astro did not display any calcium respond to KCl stimulation. A study by Foo *et al.* demonstrate KCl induced response by FBS-isolated astrocytes in contrast to immunopanned astrocytes, which upon FBS treatment respond to KCl (248).

Cholesterol and lipid homeostasis in the brain is regulated by astrocytes where one of the major transporting proteins, APOE, is predominantly produced by astrocytes. Moreover, the APOE4 isotype, which have reduced binding of cholesterol and lipids compared to APOE2/3, is a strong genetic risk factor for developing AD. Basic secretion analysis show that the astrocytic models secrete significantly more APOE compared to neural stem cells (ltNES). Transcriptomic profile linked to APOE-associated genes display similar expression patterns among the astrocytic models except for great difference by the astrocytoma cell line (CCF), which have previously been used to study APOE biology (269, 322). Finally, we wanted to compare the astrocytic models in a pharmaceutical setting optimized for high-throughput screening (HTS). Using pharmacological enhancers of APOE, assessed by a sandwich ELISA, we could optimize and demonstrate assay robustness suitable for HTS with Z' of 0.76. Thereby we could run a pilot-screen of APOE annotated compounds across all models. In summary, common LXR agonists regulated APOE secretion in CCF as previously shown (269, 322). However, overall results displayed that no annotated APOE enhancer included in this experiment induced a significant response in all astrocytic models. This indicate that hit-finding in HTS for APOE enhancer will be dependent on cellular context. Model bias was also demonstrated by Finan *et al.* which used primary fetal astrocytes in the primary screen (269), more commonly used in confirmation/secondary screens (322). The strategy led to the identification of new APOE enhancers not stimulating CCF (269), which otherwise would have been missed if the CCF was used in the primary screening. Identification of compounds in the cholesterol biosynthesis pathway having APOE enhancing effects (269) could be confirmed in our study where both NES-Astro and phaAstro responded to stimulation, while no effect was observed in the CCF.

Additionally, since the aim was to develop an astrocytic model applicable for drug screening a big emphasis was on model robustness and reproducibility. This included the removal of non-defined products, handling procedures of media and coating components, optimizing culturing timelines and passage time points, in addition to constructing a protocol for standard operating procedures providing a day to day instruction for a 6-week period. This enabled the establishment of a completely xeno-free culturing system exchanging certain components in the media and switching from the traditional coating to human recombinant laminin 521.

Protein expression profiles demonstrate the same developmental program of NES-521Astro as for NES-Astro.

All together the NES-Astro model shows biological translability and robustness of high enough quality to be applicable in a pharmaceutical setting and demonstrate better model representation in several aspects compared to traditional models used today.

6.2 Paper II

Besides studying disease phenotypes or mechanisms hiPSC derived models show great potential to provide valuable information regarding unwanted side effects from lead compounds in a secondary evaluation. We wanted to explore this possibility in a proof of concept study for the development of new plasminogen binding inhibitors (PBIs) which are used to avoid excess bleeding after traumatic injury or surgeries. First generation PBIs, including a commonly used drug TXA, have been associated with an increased incidence of seizures (305, 307). The effect has been suggested to depend on the unwanted selectivity of TXA towards inhibitory neuronal receptors $GABA_A$ and glycine (307-309). Previous development of new PBIs which did not displaying activity against inhibitory neuronal receptors used a relatively low throughput method. Additionally, this neuronal rat membrane binding assay does not provide any information regarding compound mode of action (311). Besides validating the application of hiPSC derived neurons for the evaluation of new PBIs we wanted to explore the possibility to integrate a technique enabling future high throughput screening (HTS) that provides information on mode of action. The label free technology measuring dynamic mass redistribution (DMR) by optical sensors, previously used for studying G protein-coupled receptor activity (317) is suitable for HTS purposes. As compared to astrocyte, the neuronal protocols are more well established proving expressional and functional characteristics suited for this evaluation (312, 313). Adaptation of a commercial hiPSC neuronal line with the DMR system demonstrated good viability and DMR signal. Next, we evaluated if stimulated activity of endogenously expressed $GABA_A$ and glycine receptors could be detected by the DMR-system, which previously been used for overexpressing or immortalized cell lines with high receptor expression (317, 320). Indeed, we could demonstrate concentration response curves for GABA and glycine, endogenous ligands for the $GABA_A$ and glycine receptors, respectively. The signal response could be confirmed to be dependent on $GABA_A$ and glycine receptor by stimulating with antagonist bicuculline and strychnine, respectively. Concentration response curves for antagonists GABAzine and strychnine could also be demonstrated. To evaluate assay robustness, we ran the assay at two different time points using different vials of hiPSC neurons displaying stringent results.

Additional evaluation of the assay explored the previous suggested promiscuous properties of taurine activating both the $GABA_A$ and glycine receptor at high concentrations (353). Applying taurine at increasing concentrations under co-treatment with antagonists towards the $GABA_A$ and glycine receptor demonstrate that taurine below 1mM only activate the glycine receptor while concentrations above 1mM also activate the $GABA_A$ receptor. To further explore the flexibility and granularity of the assay we investigated if we could detect and show indications

of the glycine receptor composition by applying picrotoxin, an α-homomeric glycine receptor antagonist. The result demonstrated a reduced signal of taurine stimulation at 0.375mM in the presence of picrotoxin compared to control indicating that hiPSC neurons have a heterogenous expression of α-homomeric and αβ-heteromeric glycine receptor composition.

After validating the functionality and representability of combining hiPSC neurons with the DMR technology we next looked at the claimed effect of commonly used drug TXA. The stimulation with TXA showed antagonistic effect on both the $GABA_A$ and glycine receptor indicating a potential cause for the increase incidence of seizures during TXA administration to patients. With the goal to evaluate if the assay could be used in drug developmental programs to exclude unwanted chemical properties we evaluated chemical entities from a lead series of compounds with PBI properties. We applied one discontinued compound, indicated to have effect on the $GABA_A$ receptor, and a candidate drug, AZD6564, shown to have no effect on inhibitor neuronal receptors (311). In contrast to AZD6564 we could demonstrate that the discontinued compound had an antagonistic effect on the $GABA_A$ receptor reducing the efficacy of GABA down to 70%.

In summary, the study demonstrates proof of concept that hiPSC neurons together with a HTS applicable technique can be used in the stratification of lead series compounds in drug development.

6.3 Manuscript III

Independent of the application, model representation is critical for its relevance and impact. In the light of the results in Paper I, presenting a big difference in model representation of astrocyte biology, we ask how the derivation of NES-Astro correlate with *in vivo* development. In what aspects can the NES-Astro model be used to study developmental features of human glia biology, indicated to have evolutionary significance to brain development and cognitive function, and what is the NES-Astro translability? To rely on results achieved with the NES-Astro model these are key question to be answered to build confidence in decisions made moving forward in drug discovery programs.

To thoroughly investigate the development of NES-Astro using the FHIA-protocol RNA samples were taken at day(d)0, d7, d15, d22, and d28, which the last time point represents the same stage as the evaluation in Paper I. To increase confidence in the transcriptomic patterns we performed three independent culture, technical replicates, of three independent ltNES cell lines, biological replicates, in total n=9. In addition, we used the exact same set up of n=9 at the same time points for control conditions where ltNES were cultured under maintenance conditions instead with the directed FHIA-protocol. In total this generated 81 RNA samples evaluated using RNA sequencing. Basic analysis further confirms the robustness of the FHIA-protocol presented in Paper I as well as displaying a good transcriptomic coverage corresponding to the transcriptome from fetal brain samples (354). Mathematical analysis of the transcriptomic patterns clustered genes sharing high fold change compared to control condition. Gene list enrichment analysis of these clusters demonstrated clear association to

brain and neural cell biology and development. Additionally, genes related to glutamate associated biology were significantly changed correlating with the demonstrated glutamate function of NES-Astro presented in Paper I. Intriguingly, genes related to circadian rhythm, ECM and integrin pathways are significantly regulated during NES-Astro development. These are gene classes hypothesis to regulate the stem cell niche of proliferating oRG which is highly associated to the expansion of the human neocortex (2, 13, 148). Single cell RNA sequencing has enable to categorize cellular diversity based on transcriptome identities. The top differentially express transcripts in these diversified cellular profiles can provide indications on which cell types make up a certain population of cells. Comparing single cell transcript identity profiles with NES-Astro populations during the differentiation display a transition of cellular identities also found during embryonic development. Additionally, comparing which cell identities are shared across the differentiation time points would indicate how the composition of population change over time. What we saw was that intermediate progenitors and neurons are only expressed at high levels at few time points while glia associated transcripts accumulate over time, indicating an enrichment of the glia population. To validate this enrichment of glia transcript identity we looked at the protein expression patterns of known markers important in glia biology. In accordance to transcriptomic profiles we could display a gradual increase in expression homogeneity of SOX9, NFIA, FABP7, S100B and SLC1A3.

Recently, there has been an emphasis on the importance of non-coding RNAs, and its relation to primate specific cortical complexification (13). From the transcriptomic data we could observe a strong down regulation of key regulators in the miRNA biogenesis processes which are linked to miRNAs indicated to regulate the neurogenic-to-gliogenic switch (144). To further validate the transcriptomic and protein expression profiles, demonstrating the occurrence of the neurogenic-to-gliogenic switch taking place during NES-Astro development, we performed a small RNAseq at the differentiation time points. A strong increase of the Let-7 family and downregulation of LIN28A/B previously shown to regulate gliogenic potency (144) further validate the switch taking place. Additionally, miRNAs associated to astroglia cell fate is upregulated at d28.

One of the main advantages of using hiPSC is the human origin which is important studying human specific traits. Lineage tracing studies indicate that RG and SVZ progenitors are sources of astrocytes (12, 33). Since the oSVZ, hosting a proliferative oRG population, is a structure associated with human brain development (2, 13, 148) we asked if the NES-Astro model displayed human specific developmental features. Based on the transcriptomic patterns we could observe an increased expression of oRG identity together with transcripts associated to mechanism hypothesized to regulate the stem cell niche of oRG including ECM regulation, STAT3 and notch signaling (148). The transition of oRG to acquire an astrocytic fate is not known, but some oRG enriched genes are also enriched in astrocyte (148), which we observe in the NES-Astro model together with an increase of astrocyte maturation markers (154, 175). It is intriguing to speculate that since NES-Astro show an increased transitional association to mature astrocytes, an expression profile of GFAP- /S100B+ /SLC1A2+ /SLC1A3+ and the functional maturation of glutamate calcium responsiveness the model could represent

progenitors of an immature protoplasmic astrocytes (126, 154, 175, 179). That would provide the possibility to study one of the major astrocytic subtypes using this hiPSC derived *in vitro* model.

7 Conclusions and Future Perspectives

This thesis demonstrates that hiPSC derived neural models can have a significant role in drug developmental programs. Notably, however, the model having the highest representation and translability in relation to its feasibility will be favored in attempts to deliver drugs to patients, independent on model type. Therefore, the establishment of model translability is key in order to draw the right conclusion moving forward in drug development. As demonstrated in the pilot-screen of APOE enhancers the current models used in the pharmaceutical industry will identify hit compounds with model bias. Even though validation of hits is performed in a second model the order in which you use the models, either for a primary screen or in a secondary validation stage, will affect hit identification with the risk of excluding compounds only active in one model (269).

As astrocytes are being increasingly recognized to play important roles in human neurological diseases (355, 356) there is a need for models with high human translability. Astrocytic model representation has for a long time intensively focused on astrocytes associated GFAP expression even though a large portion of astrocytes *in vivo* do not express this protein (126). Since many protocols for differentiation (242, 264), isolation (248) and cell cultures (168) are FBS-based, known to drive reactivity and GFAP expression (20, 154, 166, 248), the study of GFAP negative astrocytes, predominantly protoplasmic astrocytes, will be challenging. Improved astrocyte isolation protocols, highly preserving *in vivo* characteristics (154, 175), and more defined culturing conditions (20) still focus on the GFAP+ population. A key factor to generate hiPSC derived astrocytes representing the GFAP negative protoplasmic population is to understand the developmental origin of astrocyte heterogeneity. Moreover, both the GFAP+/- astrocyte populations are diverse in a spatial and functional manner highlighting the need for increased stratification of modeling astrocyte biology. Single cell RNA sequencing will help identify key transcriptional pathways in astrocyte development, which with even more refined RNAseq methods (176), will further help to understand astrocyte heterogeneity.

Even though astrocyte functional heterogeneity is not fully understood both neurons and astrocytes demonstrate great physiological diversity. With increased understanding of cellular stratification of the brain we can also more precisely link pathological conditions to specific cell types. As this increases the complexity of modeling it also enables more targeted drug interventional approach with plausibly lower risk of unwanted side effects. With increasing number of subspecific models hiPSC derived neural cells have a great potential role in such strategies. Both in disease mechanistic projects or as validation of potential off target effects.

There is always a limited access to human tissue, and since some of these developmental features are linked to human biology animal model representation is not sufficient (148, 154). Besides the uncertainties of being an *in vitro* model system human brain organoids possess a great potential to answer some of these questions (175). In addition, since there is a close interplay between neurons and astrocytes during their biological development (19) more complex models are needed to increase the biological translability of hiPSC derive neural models. However, this currently provides a challenge in drug development programs driven by

high-throughput screening where monolayers of homogenous cell populations are favored for its streamlined process. With technical development of single cell analysis methods including transcriptomics and proteomics, this will most likely change where HTS is run in microfluidic systems based on single cell stimulation and readout. Alternatively, drug stimulation of organoids or organs on a chip, where different cell types are affecting each other, followed by single cell analysis will help understand plausible systemic drug effects *in vitro*. As single cell analysis generates enormous amounts of data both infrastructure and data analysis need to be developed to provide scientific interpretation and impact. Artificial intelligence will most likely be a solution to this problem and might have a central role in future drug discovery.

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